Animal Welfare in Biomedical Research – Behavioural Alterations as Pain Indicators in Laboratory Mice

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This thesis is dedicated to my mother and my grandparents who supported me each step of the way.

“The welfare of animals must depend on an understanding of animals, and one does not come by this understanding intuitively it must be learned” (Medawar 1972)

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SUMMARY

Animal welfare in biomedical research—Behavioural alterations as pain indicators in laboratory mice

Assessing animal welfare is a crucial prerequisite to avoid and minimise pain, stress and suffering in animal experimentation. Considering the difficulties that still remain in the routine assessment of welfare or well-being of mice in the laboratory, we aimed to develop easy-to-use and non-invasive behavioural indicators of states that affect animal welfare adversely like pain, distress and impairment. To this end, we analysed changes in several species-typical home cage behaviours and their temporal distribution after surgery under different housing conditions as well as in a model of experimentally induced colitis.

Initial studies involved inhalation anaesthesia only or anaesthesia and a one-sided minor laparotomy with or without pain treatment (the non-steroidal anti-inflammatory drug carprofen) in male and female C57BL/6J mice. Home cage behaviour of mice was recorded with infrared sensitive cameras for up to 24 hours. Unlike classical signs of reduced well-being (body weight reduction, clinical symptoms and pain behaviours like stretching), post-experimental profiles of home cage behaviours differed significantly from those of a healthy control group. Significant changes in resting and locomotion as well as in self-grooming and climbing behaviour were seen, being on a moderate level after anaesthesia and marked after surgery compared to the control group. Although not significant, a tendency towards more marked changes was seen in surgery without pain treatment compared to surgery with pain relief. While resting and locomotion were decreased as well as increased at several time points contrary to their circadian rhythm, self-grooming was increased independently of the time of observation. Our results highlight the requirement for knowledge of species-specific circadian rhythms of behaviours as well as the importance of determining the appropriate time of day for behavioural assessment.

A distinct reduction in nest complexity was also observed after surgery or anaesthesia. While anaesthesia led to a moderate decrease of nest complexity, fewer than 10% of animals had identifiable nests after surgery. This massive decrease in nest complexity could not be alleviated in a significant manner by analgesia.

However, changes in burrowing performance proved to be sensitive to the impact of anaesthesia, post-surgical pain and the analgesic treatment used. A distinct gradation in burrowing performance, ranging from an intermediate level after anaesthesia to a distinct prolongation of latency after surgery, could be observed. Moreover, analgesia after surgery was associated with a significantly earlier onset of burrowing than after surgery without pain relief, reaching a level comparable to latencies after anaesthesia only. The prolongation of latency to burrow might provide hints as to the duration of post-surgical pain and the progression of recovery from surgery. In a subsequent experiment the burrowing test was also used successfully to detect the onset of inflammatory bowel disease in a mouse model at an early stage.

In further studies our behavioural parameters were used to reveal differences in recovery of animals housed under different post-surgical housing conditions. Even though no distinct negative effect of individual housing after surgery could be observed, a tendency towards better recovery of behaviours classified as positive was found in pair-housed mice. Animals housed in a new clean cage after surgery showed signs of reduced well-being, agitation and restlessness. These observations may hint that animals cope better with surgical stress when housed in groups and in their familiar environment. The post-surgical transport to a new and clean cage and

individual housing might be additional stressors after an exhausting event and may affect fast recovery detrimentally.

In summary, assessment of general changes in animal well-being was possible with all the behavioural indicators analysed. While most home cage behaviours and the scoring of nest complexity, were found not to be sensitive enough to indicate efficiency of the analgesic treatment in a reliable manner, burrowing performance proved to be a sensitive tool to assess pain, stress, and impairment as well as analgesic effect in two clinical settings and under different housing conditions typically occurring in animal experimentation.

In particular, assessment of nest complexity scoring and the burrowing test may act as positive welfare indicators and can be easily adapted to laboratory routine. These behavioural indicators offer assessment criteria in a wide range of clinical situations and experimental models associated with pain, stress and suffering, thus providing a basis with which to fulfil legal, ethical and scientific obligations for the laboratory animal. These indicators help to adjust pain management and strain-reducing measures and to refine methods as well as to assess the impact of different procedures and phenotypes on animal well-being and welfare to determine the prospective and retrospective severity grades required by legislative authorities.

ZUSAMMENFASSUNG

Tierschutz in der biomedizinischen Forschung— Verhaltensänderungen als Schmerzindikatoren bei Labormäusen

Die Beurteilung des Wohlbefindens von Versuchstieren ist ein entscheidendes Werkzeug für den Tierschutz in der biomedizinischen Forschung, da sie die Grundlage zur Vermeidung und Minimierung von Schmerzen, Distress und Leiden bildet. Im Laboralltag zeigt sich die Erfassung des physiologischen und psychologischen Status insbesondere kleiner Nagetiere allerdings als anspruchsvoll und für das am häufigsten genutzte Labortier, die Maus, nicht immer zufriedenstellend realisierbar. In Anbetracht dieser Schwierigkeiten, wurden in den hier vorgestellten Studien einfach einzusetzende, nicht-invasiv messbare Verhaltensindikatoren entwickelt, die auf Zustände wie Schmerz, Stress und Belastung hinweisen, welche das Wohlbefinden von Tieren reduzieren können. Dazu untersuchten wir Veränderungen in verschiedenen speziestypischen Verhalten im Heimkäfig und deren zeitliche Entwicklung nach einem chirurgischen Standardeingriff unter verschiedenen Haltungsbedingungen und in einem Modell experimentell induzierter Kolitis.

C57BL/6J Mäuse, beider Geschlechter, wurden einer Inhalationsanästhesie oder einer Inhalationsanästhesie in Kombination mit einer einseitigen Laparotomie, jeweils mit oder ohne Schmerzbehandlung mit dem nicht-steroidalen Entzündungshemmer Carprofen, unterzogen. Das Verhalten der Tiere wurde dann im Heimkäfig mit Infrarot sensitiven Kameras für bis zu 24 Stunden aufgezeichnet. Im Gegensatz zu klassischen Zeichen reduzierten Wohlbefindens (Körpergewichtreduktion, klinische Symptome und Schmerzverhalten wie stretching), unterschieden sich die post-experimentellen Verhaltensprofile deutlich von denen einer unbehandelten Kontrollgruppe. Signifikante Veränderungen in der totalen Dauer von Ruhe-, Putz- und Fortbewegungsphasen sowie im Kletterverhalten konnten beobachtet werden. Diese zeigten sich moderat nach Anästhesie und ausgeprägter nach dem operativen Eingriff, wobei eine, wenn auch nicht signifikante, Tendenz zu stärkeren Veränderungen in der OP-Gruppe ohne Schmerzbehandlung als in der OP-Gruppe mit Schmerzbehandlung beobachtet werden konnte. Während Ruhe- und Fortbewegungsdauer zu bestimmten Zeitpunkten entgegengesetzt ihrer normalen zirkadianen Rhythmik erhöht und an anderen reduziert waren, zeigten die Tiere post-experimentell mehr Putzverhalten unabhängig vom Zeitpunkt der Beobachtung. Unsere Ergebnisse betonen die Notwendigkeit einer guten Kenntnis arttypischer zirkadianer Rhythmen sowie die Bedeutung des gewählten Beobachtungszeitpunkts für verhaltensbiologische Untersuchungen dieser Art.

Nach Anästhesie oder operativem Eingriff zeigte sich zusätzlich eine Abnahme der Komplexität, der von den Mäusen gebauten Nester. Während Anästhesie zu einer moderaten Abnahme der Nestkomplexität führte, verfügten nach dem operativen Eingriff weniger als 10% aller Mäuse über erkennbare Nester. Diese massive Abnahme der Nestbauaktivität konnte durch die Gabe von Schmerzmittel nicht signifikant gemildert werden.

Veränderungen im Tunnelgrabverhalten erwiesen sich hingegen als sensitiv gegenüber dem Einfluss von Anästhesie, post-operativem Schmerz und auch der Schmerzbehandlung. Eine klare Abstufung der Latenzzeiten wurde nach den experimentellen Eingriffen verschiedener Schweregrade beobachtet. So verlängerte sich die Latenz verglichen mit der unbehandelten Kontrollgruppe nach dem operativen Eingriff stärker als nach der Anästhesie. Dieser Effekt, hervorgerufen von der schmerzhaften Operation, konnte durch die Gabe des Schmerzmittels signifikant auf das Niveau nach Anästhesie reduziert werden. Die Verzögerung der Grabaktivität könnte zusätzlich ein Hinweis auf die Länge post-operativer Schmerzen und die Rekonvaleszenz nach der

Operation sein. In einem anschließenden Experiment konnte dieser Verhaltenstest erfolgreich eingesetzt werden um den Beginn einer entzündlichen Darmerkrankung im Mäusemodell frühzeitig anzuzeigen.

In weiteren Studien nutzten wir unsere Verhaltensindikatoren, um den Einfluss verschiedener Haltungsbedingungen auf die post-operative Rekonvaleszenz von Mäusen zu untersuchen. Auch wenn kein signifikant negativer Einfluss kurzzeitiger Einzelhaltung nach operativen Eingriffen nachgewiesen werden konnte, zeigte sich eine Tendenz zur schnelleren Erholung verschiedener, als positiv eingestufter Verhalten in der Gruppenhaltung. Mäuse, die nach der Operation in einen neuen, sauberen Käfig verbracht wurden, zeigten verglichen mit Mäusen, die in ihren vertrauten Käfig zurückkehrten, Zeichen von reduziertem Wohlbefinden, Beunruhigung und Ruhelosigkeit. Diese Ergebnisse sind Hinweise darauf, dass Mäuse post-operativen Stress besser bewältigen, wenn sie in ihrem vertrauten räumlichen und sozialen Umfeld gehalten werden. Der post-operative Transport in einen neuen Käfig sowie die post-operative Einzelhaltung können als zusätzliche Stressoren nach einem strapaziösen Eingriff angesehen werden und scheinen negativen Einfluss auf eine schnelle Rekonvaleszenz zu haben.

Zusammenfassend war eine Beurteilung genereller Veränderungen im Allgemeinzustand der Tiere mit allen untersuchten Verhaltensindikatoren möglich. Aufgrund ihrer geringen Empfindlichkeit eigneten sich die meisten Verhaltensindikatoren einschließlich der Beurteilung der Nestkomplexität allerdings nicht für einen eindeutigen Nachweis der analgetischen Behandlung. Schmerz, Stress und Belastung sowie die analgetische Wirkung konnten hingegen durch die Beurteilung des Tunnelbauverhaltens robust in zwei in Tierversuchen typischerweise auftretenden klinisch relevanten Zuständen und unter verschiedenen Haltungsbedingungen nachgewiesen werden.

Insbesondere die Bewertung der Nestkomplexität sowie der Tunnelbauaktivität eignen sich als Indikatoren für gutes Wohlbefinden und können in der Laborroutine einfach angewendet werden. Sie bieten Beurteilungs-kriterien für ein breites Spektrum von Experimenten und klinisch relevanten Zuständen, die mit Schmerz, Stress und Leiden verbunden sind, damit liefern sie eine Grundlage die gesetzlichen, ethischen und wissenschaftlichen Verpflichtungen gegenüber dem Labortier zu erfüllen. Sie helfen Schmerzbehandlungsregime und belastungsmindernde Maßnahmen anzupassen, experimentelle Methoden zu verbessern, wie auch den Einfluss verschiedener Prozeduren und Phänotypen auf Wohlbefinden und Tierschutzaspekte hin zu bestimmen und die vom Gesetzgeber geforderten prospektiven und retrospektiven Schweregrade zu definieren.

INTRODUCTION:

ANIMAL WELFARE IN BIOMEDICAL RESEARCH —BEHAVIOURAL ALTERATIONS AS PAIN INDICATORS IN LABORATORY MICE

Animal experimentation

The number of vertebrate animals used in biomedical research, teaching and testing worldwide was estimated at 75–100 million in 2005; 44% of these animals are assumed to be mice (1). The relative proportion of mice as laboratory animals used for research in 2005 in the European Union and Switzerland was even higher, at 53% and 60% respectively (2, 3) (see also figure 1).

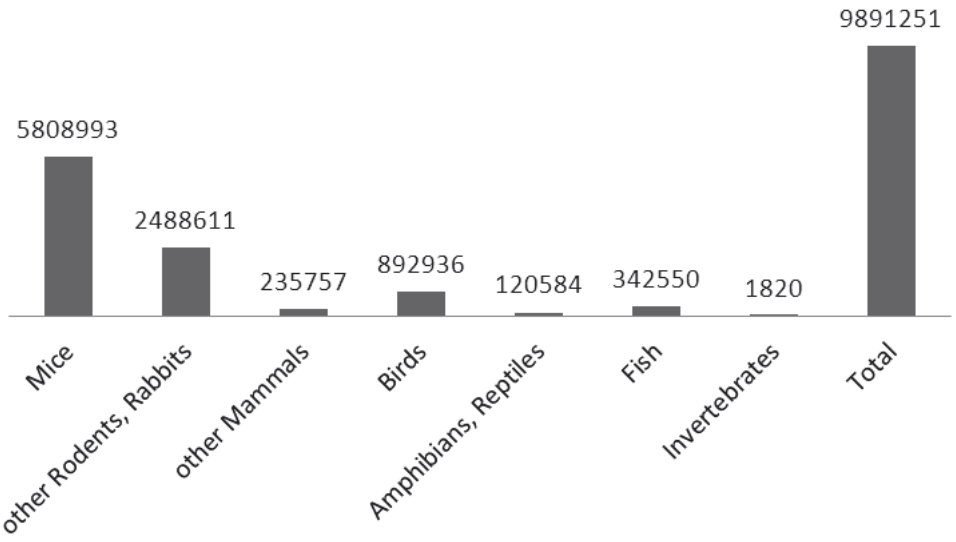


Figure 1: *Total numbers of animals used in experiments between 1997 and 2010 in Switzerland (Source: Federal Veterinary Office).*

Recent years have seen a further increase in the numbers of mice used in Switzerland, with the current high level possibly due to the availability of various genetically modified models and the increased use of such lines in research. While only 40,000 genetically modified mice were used for experiments in Switzerland in 1997, this number increased to approximately 130,000 in 2011 (2). As animals used for breeding only are not counted, the actual number might be even higher.

Laboratory animal science and the 3Rs

The increasing demand for high quality animal models together with the more critical public view taken of the use of animals for experimental purposes led to the development of laboratory animal science in the 1950s. Laboratory animal science is a multidisciplinary field contributing to improving both the quality of experiments in which animals are used and animal welfare (1). The guiding principles—first formulated by Russell and Burch

(4)—encompass the three concepts of Replacement, Reduction and Refinement (termed the 3Rs) and provide a systematic basis for humane procedures in experimental science.

The approach of Replacement is to use non-sentient organisms like microorganisms and plants or artificial materials and computer techniques rather than highly developed animals. In vitro techniques with cell cultures from animal tissues can be defined as "relative replacement" procedures, since the experimental materials used are still derived from animals.

Reduction means obtaining the best quality and most precise information with the smallest possible number of animals. Good experimental design, including statistical concepts and standardising in terms of genotype, hygiene status and animal housing deliver reliable results, and eliminate the need for repetition of tests.

Refinement, defined as any change in protocol that reduces the incidence or severity of pain and distress experienced by the animals, is the most subtle approach. This principle extends beyond the legal and ethical demands for minimisation of suffering as procedure refinement may also have scientific benefits. There is evidence that animals whose well-being is compromised are often physiologically, behavioural and immunologically abnormal and experiments using such animals may reach unreliable conclusions (5).

Legislation in the European Union and Switzerland

In most countries, the general public demands the ethical treatment of animals used for scientific purposes. Many countries therefore have regulations for the breeding, housing and use of laboratory animals included in their animal protection laws or have formulated special animal testing regulations, laws or guidelines that permit and control the use of animals for scientific experimentation.

In the European Union, "Directive 2010/63/EU on the protection of animals used for scientific purposes" (which updates and replaces Directive 86/609/EEC) came into force in November 2010 (6). Directive 2010/63/EU includes explicit regulations based on the three principles of the 3Rs and establishes these as legal obligations within the European Union (e.g. Chapter I: General Provisions, Article 4).

Likewise, Swiss authorities have in recent decades formulated far-reaching and detailed laws and guidelines concerning the breeding, housing and use of laboratory animals. While Article 4 of the Animal Welfare Act of 2005 (7), revised in 2012, formulates the basic principles to ensure well-being, the Animal Welfare and Animal Experimentation Ordinance formulated in 2008 and 2010 (8, 9), respectively, regulate the more practical aspects of breeding, housing and use of animals for experimentation regarding the prevention and reduction of pain, distress and suffering in laboratory animals (e.g. Section 4 of the Animal welfare Ordinance, Article 135: Performance of experiment). Adherence to these regulations, as well as the strict use of “methods in particular to limit pain, suffering, harm, fear or other negative effects on the welfare of individual animals (Animal welfare Ordinance, Article 141)” are the conditions that must be met in order to receive a license for animal experiments in Switzerland.

With these regulations, both the European Parliament and Council and the Swiss authorities (Swiss Federal Assembly, Swiss Federal Council and Federal Veterinary Office) aim to ensure good animal welfare for laboratory animals. They emphasize not only the importance of reducing pain, distress and suffering by choosing refined breeding, housing and experimental procedures, and the importance of anaesthestic and analgesic management for animals possibly experiencing pain, suffering and distress, but also highlight the significance of

the assessment and quantification of pain, distress and suffering as well as the evaluation of efficacy of pain-, distress- and suffering-reducing methods (Directive 2010/63/EU Chapter I: General Provisions, Article 4: Principle of replacement, reduction and refinement, Chapter III: Procedures, Article 13: Choice of methods and Article 14: Anaesthesia; Swiss Animal welfare Ordinance Section 4, Article 135: Performance of experiment).

In addition, in many countries, including the countries of the European Union and Switzerland, it is mandatory to grade, prospectively and retrospectively, the level of discomfort and harm inflicted by the experiments conducted (2010/63/EU, Article 15 and 39; (10, 11)). Again, the essential prerequisite of this practice is of course the reliable assessment of pain, distress and suffering in animals.

The new Swiss Animal Experimentation Ordinance additionally acknowledges the increasing number of genetically modified animals used in research and the possible constraints these animals can suffer. An animal line, strain or mutant might be called strained when they display a significant clinical pathological phenotype as a result of genetic predisposition and therefore may experience pain or suffering, show damage, live in fear or suffer another form of radical alteration of its appearance or its capabilities (Animal Welfare Ordinance, Chapter 1: General provisions, Article 2 Terms). To address concerns regarding these animals, Section 4 of the Animal Experimentation Ordinance specifies the recording and documentation of constraints and the reporting procedures for constraints.

Animal welfare

Although animal welfare is a common term that is used extensively in international legislation and in concepts like the 3Rs, there is no consensus regarding its meaning (12). Undoubtedly, the basic needs of an animal must be met to maintain a state of physical and psychological balance or homeostasis. If circumstances do not permit such needs to be satisfied, the result may be reduced well-being with associated somatic or psychological pathologic changes. The consideration that not only absence of disease and injury but also mental well-being are prerequisites of good welfare is not new (13). A minimal standard for the maintenance of good welfare in farm animals known as the five freedoms was published in 1965 by the Brambell Committee (14). Already at that time, this document considered the absence of negative affective states as an important component and considered not only physiological but also behavioural needs. Behavioural needs induce behavioural patterns with direct or indirect consequences. For example, a hungry animal will show eating behaviour, which has the direct consequence of satiation, while other behaviours will have more indirect long-term consequences. This is the case when the motivation to display a behaviour is governed by the display itself rather than to meet short-term needs. These behaviours are self-rewarding via, for example, the release of endorphins. Being deprived from highly motivated behaviours is a severe stressor that reduces animal well-being (15, 16).

Modern concepts of animal welfare emphasize that good welfare is not simply the absence of negative experiences, but rather is primarily the presence of positive experiences such as pleasure as well as the capability to cope with environmental challenges. Unfortunately, little is known about the factors that affect psychological well-being, and studies of negative emotions are generally more numerous than studies measuring positive emotions, especially in laboratory animals (15, 17). The latter approach deals with an animal's ability to cope with its environment and the possibility of a certain control over its life that leads to predictability and controllability. These are seen by some authors as key factors of animal well-being (17, 18).

Altogether, considering the above, it is obvious that welfare and well-being are multifaceted, and factors affecting them are interactive and interrelated. An animal's well-being or quality of life is its internal somatic

and mental state, which in turn is determined by cognition or affect and motivational state, and the responses to internal and external stimuli (12). Therefore welfare can be defined as a state of harmony between an individual and its environment and as the balance between positive and negative affective states (19, 20). However, factors that determine welfare are poorly understood, and means of assessing welfare in its entirety have yet to be validated (12). For this reason, the assessment of important designators and major factors that may affect animal welfare, such as pain, distress or suffering, which than can be combined in a holistic way becomes an integral part of welfare assessment. Pain, distress and suffering are terms basically describing states of the human mind. Transferring these definitions to comparable states in animals is a complex task. Nevertheless, researchers and caretakers responsible for the well-being of laboratory animals in breeding, housing and in experiments must be familiar with the concepts of pain, distress and suffering, and know how to recognize, assess, control and, preferably, to prevent these experiences in their animals. Even though the three terms are described separately in the following, it is important to keep in mind that there are interactions between these three states.

Pain, distress and suffering in laboratory animals

Pain

The International Association for the Study of Pain (21) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential damage". The sensory part of this phenomenon is nociception and depends on specifically dedicated receptors—so-called nociceptors—and associated pathways. Nociceptors are nerve cell endings that transduce a variety of stimuli into receptor potentials that trigger afferent action potentials (22, 23). Nociceptive nerve cell axons enter the spinal cord and project to structures in the brain stem and fore brain. A number of different brain structures and a diverse network of neurons with a complex pattern of connections are then involved in the processing of pain and the resulting behavioural or cognitive responses (22, 23). While some components of the system mediate sensory discriminant aspects, others convey information about affective-motivational aspects like the unpleasantness, fear and autonomic activation that accompany exposure to a painful stimulus (23). The above definition of pain also emphasizes that pain is an experience in a conscious organism (21). In contrast to nociception, the emotional component of pain is especially difficult to prove in non-human animals and remains a topic of debate.

Pain is a natural and protective experience that may, for example, act as a warning of danger, which can lead to defensive mechanisms and be linked with responses that help the animal cope with injury. Nevertheless, there are also many situations (e.g. in chronic cancer pain), where pain loses its protective function or pain develops pathological forms, such as chronic neuropathic pain (e.g. phantom limb pain) that have detrimental consequences for the organism (24, 25). When tissue is damaged or traumatised, the release of chemical agents like inflammatory mediators may additionally cause excitation of nerve endings. These can lead to hyperalgesia—a state in which stimuli normally perceived as slightly painful are perceived as significantly more painful. Another consequence of nerve damage can be allodynia—an increase in the excitability of neurons following high levels of activity in the nociceptive afferents—resulting in pain following a stimulus normally not perceived as painful (22, 23). But non-pathological pain can also have serious consequences as pain can slow recovery from surgery, reduce food and water consumption, interfere with normal respiration, reduce "self-maintenance" behaviour or lead to muscle spasm, atrophy and different endocrine and metabolic changes (22).

Besides the classification of pain in pathological and physiological forms, pain can occur in a multitude of ways and originate from diverse sources. Pain may be acute, lasting for seconds to days, or chronic, lasting for weeks,

months or even years. Pain may be visceral or somatic, inflammatory, post-surgical or ischaemic. Additionally, the pain sensitivity of particular tissues and organs can be very variable (22-24).

Stress, eustress and distress

Kopin (26) describes stress "as a state in which expectations—whether genetically programmed or acquired—do not match current or anticipated perceptions of the internal or external environment". It is very likely that stress responses have evolved because they were advantageous in natural selection as they provide a means to anticipate and react rapidly to external and internal threats to the individual. These responses may therefore help to preserve homeostasis, which describes all aspects of physiology that maintain life, by short-term adjustments to several systems (27, 28). Stimuli that are not inherently harmful or initiate responses that benefit the animal by maintaining homeostasis induce a state that is called eustress. Eustress can be considered normal adaptive and activities that help an organism to handle threats satisfactorily contribute to well-being (18, 28, 29). But when an animal cannot successfully adapt, cope, or habituate to the given, not necessary harmful, situation and resistance fails due to inadequate, inappropriate, or excess activation of the coping systems, serious physiological and psychological damage may result (27, 28, 30-33). This condition, often associated with anxiety, fear, frustration, anger or depression, is called distress (34).

There are no simple physiological or behavioural criteria to mark the point at which an animal becomes distressed but rather there is a continuum of stress to distress, which can be beneficial at one end but cause severe abnormal physiological changes at the other. The concept of allostasis integrates the different stress responses and emphasises the dynamic character of these fundamental processes (figure 2). Allostasis is described as an active process of maintaining or re-establishing homeostasis, as "maintaining stability through change" (28, 32).

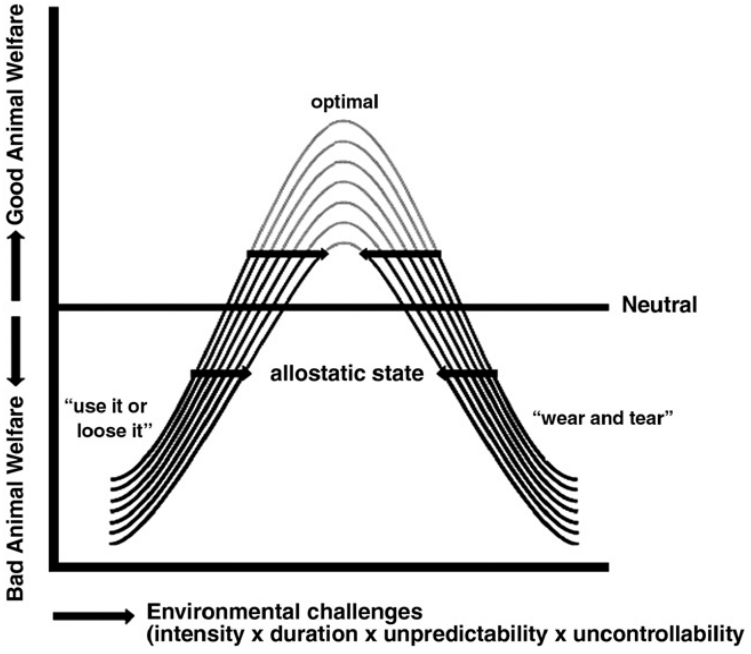


Figure 2: *The concept of allostasis. Animal welfare is shown in relation to environmental challenges (inverted U-curve). The absence of, or chronic exposure to, environmental challenges produces a state of chronic deviation of the regulatory system from its optimal operating level. Inadequately low (very low or zero) allostatic load or high allostatic load are at the basis of many stress-related pathologies (18).*

A complex of general and integrative responses by the central and autonomic nervous system, neuroendocrine system, as well as target organs of these systems, is involved in the actual stress response (35, 36). There are two important and well-known stress pathways. First, the corticotrophin-releasing hormone activation of the hypothalamic pituary-adrenal axis (HPA)—a major part of the neuroendocrine system that controls reactions to stress and regulates many body processes. Second, the sympathetic system, which has its antagonist in the parasympathetic system. These divisions are always active at some level but the sympathetic division mobilizes the body's resources for dealing with challenges, while parasympathetic activity predominates during relative quiescence, so energy can be restored (23, 24). All these systems induce a number of neural, biochemical, metabolic, endocrinologic, immune, and behavioural responses (36).

Suffering

Suffering is an unpleasant state of mind that disrupts the quality of life. It is not identical to, but might be a consequence of, pain, illness, injury, hunger, distress and emotional numbness (e.g. extreme boredom). Physical pain, for example, may result in suffering if of sufficient intensity or duration, or both. The greater the intensity, the less time is needed for pain to lead to suffering. Suffering is reached when pain exceeds a level beyond the pain tolerance threshold that the animal is able to cope with (24, 34, 37).

Assessment of pain, distress and suffering

As described earlier, the factors that determine welfare are poorly understood (12). This is especially true with regard to the mental well-being of animals. Assessing welfare in animals lacking language is a complex problem as it is impossible to investigate the emotional state directly, only to draw inferences from indirect measures. Therefore experimental results can give indirect evidence but no causal proof is possible.

This is also true for a challenging problem in animal experimentation, i.e. the assessment and management of pain in laboratory mice. Because obvious changes after invasive procedures and analgesic treatment are difficult to see in mice, the benefit of pain treatment is not obvious to care-takers and researchers. This may lead to an underuse of analgesics (38). This is a problem that occurs not only in laboratory animals but also in pet animals; one survey revealed that only 23% of small animals received pain treatment after surgery (38, 39).

While nociception can be measured, pain as an emotional perception can only be inferred. As we cannot ask animals about their pain, distress or suffering, we rely on analogies with ourselves to identify situations that are likely to be painful or stressful. The accurate observation of changes in such situations can lead us to useful assessment parameters. The same approach is used in pre- and non-verbal children, in whom pain can often be assessed using behavioural and physiological parameters (40). In animal experimentation, the reversion of such parameters to normal after the administration of analgesic or anxiolytic drugs provides good evidence that an animal may have been in a state of pain or fear.

In recent years there has been a rapid increase in information on how to estimate pain in rodents. The following sections summarise assessment parameters for factors contributing detrimentally to animal welfare available to date, with a focus on pain indicators.

Analgesiometry

Analgesiometry measures acute pain or nociceptive responses via short reactions and reflexes to determine, for example, the efficiency of analgesics. In general, brief mechanical, electrical or thermal impulses are used as

painful stimuli (22, 41). Examples of classical tests are “hot water tail immersion” (42), “acetic acid writhing” (43), “radiant heat tail flick” (44), “hot plate” (45) or “plantar” test (46). Responses (e.g. rapid withdrawal) to these brief stimuli are linked predominantly to the reflex arc and are organized by centres that are relatively low within the hierarchy of the CNS, i.e. basic motor responses. Therefore, the perception and emotional experience of pain might be missing (40). As pain in the clinical situation is more multifaceted, and pain behaviours are organized by higher centres, analgesics that show efficiency in these tests may fail in clinical pain (41, 47). Nevertheless, the hypersensitivity or hyperalgesia and allodynia that can follow tissue damage can be assessed and quantified in a reliable manner with these tests.

Clinical and physiological parameters

Physical health is a very important criterion for welfare. It is obvious that disease and injury are major causes of pain and suffering; nevertheless animals may still suffer despite an external appearance of general health (13). Therefore, assessment of an animal’s health and welfare state using clinical signs has to be undertaken with caution.

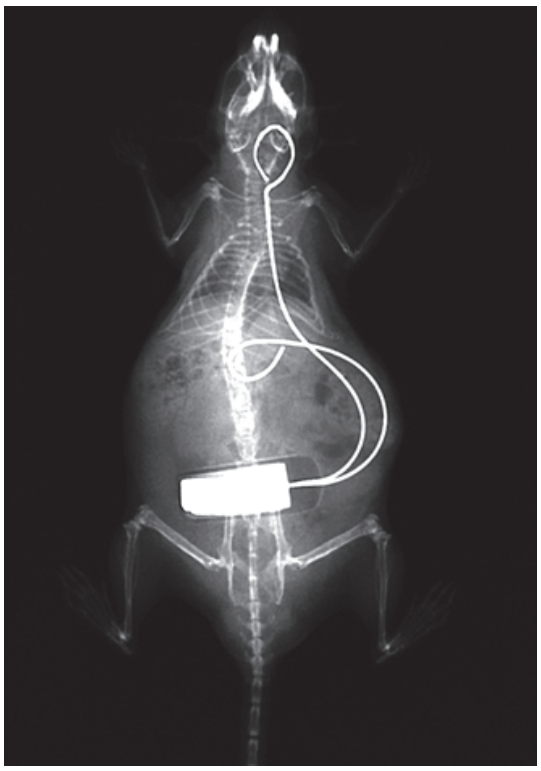
In laboratory routine, small rodents undergo regular short-term clinical observations. Standard routines include a check of the outer appearance and posture of the animal. Ungroomed, rough or dirty fur (figure 3), piloerection and eye and nose openings clotted by secretion are changes that correlate with impaired well-being and disease (34, 48). Abnormal posture, sunken flanks, a hunched back or trembling and spasms are also reliable signs of severely reduced well-being and general condition (34, 48). These very obvious changes in appearance and posture are correlated with severe health states or even moribund animals, and for decades have also been interpreted as correlates of pain or distress (34, 48). However, mild or moderate pain or suffering cannot be assessed reliably by observing such signs (40).

Figure 3: *Mouse with several classical clinical signs of reduced well-being: Ungroomed fur, hunched back and sunken flanks.*

Other clinical signs correlating with pain and disease are changes in breathing pattern, dilation of pupils, and a change in body temperature (22, 49). But these symptoms are difficult to assess in standard cage site observations of small animals.

Standard health monitoring parameters that are easy to obtain are body weight and food and water intake. A predefined reduction in these values is often used for the determination of humane endpoints. Nevertheless, these parameters are retrospective, relatively unspecific and single housing may be needed to obtain such data (e.g. (40, 50, 51)).

Pain, distress and suffering are often accompanied by responses of the hypothalamic pituary-adrenal axis and sympathetic system. The target organs of these systems release catchelomines like adrenalin and noradrenalin or corticosteroids. Levels of these hormones or their metabolites in blood, serum or faeces can be used to measure the impact of pain or stress. But sampling has to be performed very fast, especially in the case of blood and serum, otherwise only the acute stress response to handling is measured (34, 50). It has to be kept in mind that changes in hormonal factors are complex and that not all aversive stimuli result in a measurable response. Therefore hormone concentrations cannot be used as definitive measures of pain and stress (17).



The same is true for heart rate, heart rate variability, respiratory rate and body temperature, all of which may provide an estimation of the sympathetic and parasympathetic components of autonomic system activity and can be measured with telemetric devices (52, 53) (figure 4). For example, although Arras et al. (54) could show that post-surgical pain elevated heart rate, it has to be kept in mind that these transient activations of the stress axes are coincident with any kind of emotionally triggered activity, positive or negative (15). Therefore it may be difficult to discriminate between pain and stress or other states of arousal or excitement.

Figure 4: *Radiograph showing location of an implanted telemetry transmitter. The body of the transmitter is positioned in the abdominal cavity (55).*

Compromised immune status (15) and morphological changes like adrenal gland enlargement and gastric ulceration have been observed as long-term results of on-going pain and stress. But these symptoms also are very unspecific and can often only be detected post mortem (17, 56, 57).

Behavioural parameters

Animals do not behave like humans in pain or distress and different species have species-typical pain and stress behaviour. Carnivora for example normally show obvious pain behaviour, while pain signs in rodents are more subtle (22).

This condition hampers the use of behaviour as a pain or distress indicator, even for laboratory animal specialists and veterinarians (58). Additionally, the assessment of behavioural signs has been criticised as being subjective and complex, with results difficult to interpret and quantify. But recent publications show the potential and

promising use of behavioural indicators in the clinical assessment of laboratory animal pain as well as in basic pain research.

Simple avoidance or defence reactions like reflex withdrawal after palpation or manipulation have been used for decades to assess pain in animals. While these examinations might be useful in rats, because of their small body size mice are more difficult to palpate.

Aside from these obvious reactions rats and mice show a multitude of more subtle signs of pain, like abnormal posture, gait or speed, guarding behaviour, increased weight bearing on one limb, pulling toe nails and licking, biting, chewing, scratching of the injured body part (24, 59). To assess these parameters, careful observation or automated gait analysis are essential.

Automutilation or self-harming is a drastic behaviour and basically restricted to neuropathies. It might also be a result of paraesthesia and dyesthesia and not necessarily pain per se (59).

There are several more complex but relatively unspecific behavioural changes that are correlated to painful and stressful situations. Aberrant or atypical behaviours like stereotypies—the excessive display of a fixed sequence of behavioural elements—may be caused by frustration, lack of stimulation or stressful conditions. They are suggested to be a way animals cope with and adapt to stress (20, 56). More short-term behavioural consequences of stressful conditions might be freezing behaviour, increased arousal, vigilance, attention, or even stress-induced endogenous analgesia (56).

Alterations in social behaviours and circadian rhythm are easy to miss during cage site observations but are reliable signs of reduced well-being. Unusual social withdrawal as well as aggression towards cage mates or humans is the social result of stressful and painful conditions in many species (40). Changes in locomotor activity, like apathy, reduction in spontaneous activity, or hyperactivity and restlessness as well as a disturbed circadian rhythm give reliable hints as to pain or stress experienced by animals (22, 40, 60-62). Unfortunately, these changes are difficult to detect during short-term observations. Open field tests, running wheel activity recordings or video recordings of home cages that can be analysed with automated systems like *Home Cage Scan* (63) or *Ethovision* (64) might help to facilitate such observations. Abnormalities in sleep patterns can be determined by changes in electroencephalogram and electromyogram activity (59).

Regarding the assessment of pain, more specific signs such as pain-specific aberrant behaviours, facial expressions and vocalisation have been described by several authors in the last decade. While the above-mentioned complex behaviour measures might be affected by several external factors, these specific parameters are claimed to be intrinsic indicators of pain. Mice and rats with post-surgical and abdominal pain show aberrant behaviours like, for example, pressing the abdomen to the floor, with or without hind paws stretched backwards or a partial loss of balance (65-67). A recent publication showed that mice and rats grimace when in pain after several painful procedures although the changes in facial expressions are more subtle than in other species (68). Ultrasonic and audible vocalisation has been shown to occur in rats in acute pain and stress (e.g. (69, 70)), although use of these markers to measure chronic pain is controversial (71-74). In mice, audible and ultrasonic vocalisation may be associated with positive states like reproduction, e.g. songs of male mice (75), and negative states, e.g. vocalizing of mouse pups when cold or distressed. While Sevcik and co-workers could show that palpation induced vocalisation in mice suffering from late-stage pancreatic cancer (48), Williams and co-workers could not correlate audible and ultrasonic vocalisation to painful procedures like tail snip. These latter authors concluded that vocalisation in mice is not a good parameter, at least of acute pain (76). All described pain-

specific parameters require transferring the animal to an observation chamber and, in the last two cases, also specialised recording equipment.

While all the general and specific parameters listed above can be described as spontaneously occurring behaviours, experimental setups might also help in assessing pain and distress. Giving animals the opportunity to self-administer analgesic drugs can give insights into the strength and duration of painful states (77, 78). Also learned avoidance reactions might point to the aversiveness of certain conditions (34, 37).

Despite observing aberrant behaviours and signs of reduced well-being and pain positive welfare indicators can be assessed (15). The effect of housing condition on well-being, for example, can be assessed by preference tests or measuring anticipatory behaviour like the increased activity prior to an announced situation (15, 79). Other positive welfare indicators, observed in the home cage, can give an overview of the animal's mental state. These behavioural indicators can be separated in three categories: (i) Indications of pleasure, (ii) performance of luxury behaviours or (iii) behaviours that support ability to cope with challenge. Luxury behaviours like play are key factors to observe. These behaviours are normally the first behaviours that get lost during challenging situations. Display of these behaviours indicates that the animal is not deprived of an important source of pleasure and important needs are being met (15). In adult mice play is hardly seen but also other behaviours for example affiliate or sociopositive behaviours like allo-grooming may be displayed only when conditions are safe (15). In general the display of behavioural diversity is a sign for good welfare (80) while absence of species-specific behaviours might be an indicator of pain or stress. A profound knowledge of the "norms" of mouse behaviour as well as complex and extensive observation is prerequisite of these approaches.

Studies

An essential prerequisite to managing pain and monitoring the efficiency of analgesic therapy optimally is the ability to recognise the presence of pain and to assess its severity. Concerning the most widely used laboratory animal species—the mouse—there has been a lack of non-invasive methods to assess mild-to-moderate, lasting pain due to biomedical experimental interventions, a fact that has aggravated treatment of this kind of pain. While the withdrawal of pain relief is ethically reprehensible and might be legally excluded, the overdosing of drugs with side effects, such as the food intake reduction caused by opioid treatment or the gastrointestinal and renal toxicity of non-steroidal anti-inflammatory drugs, may also lead to a severe reduction in well-being (60, 81). Until now most pain management regimes in mice rely on anecdotal experience, rather than on evidence-based protocols that can be adapted to the needs of the individual.

Aiming to meet the need for reliable and easy to use measures of pain as well as distress and suffering, we conducted a series of experiments to identify behavioural pain and stress indicators for laboratory mice. In contrast to most physiological and clinical parameters, behaviour can be observed easily in a non-invasive manner and can provide a sensitive correlate of the internal state of an animal. We therefore analysed changes in several species-specific home cage behaviours and their temporal distribution in different painful and stressful states as well as under different housing conditions.

Species-specific behaviours as pain indicators

In the first study we assessed pre- and post-surgical behavioural profiles and the temporal distribution of changes in these profiles to search for behavioural indicators of pain and stress (figure 5). In the subsequent three studies we used two highly motivated, species-specific home cage behaviours, burrowing and nest building. Both

behaviours can be described as luxury behaviours, at least in the laboratory environment, because the construction of dens and nests loses its significance in the conditioned and standardised laboratory environment. Nevertheless both behaviours seem to be highly motivated, and nest building, at least, is accepted as a basic need for laboratory rodents among experts (82-85) and in Swiss and European legislation (6, 86).

Figure 5: *Observation cages. Animal behaviour was recorded in the home cage with infrared sensitive cameras.*

Chapter 1 Impact of inhalation anaesthesia, surgery and analgesic treatment on home cage behaviour in laboratory mice

Depending on the circumstances, individuals in stress or pain may show not only a decrease in certain behaviours but also an excessive display of otherwise normal home cage behaviours like locomotion or self-grooming (15). Our initial study determined the impact of short inhalation anaesthesia (sevoflurane, figure 6) and minor surgery with or without pain treatment (carprofen, 5 mg/kg) on spontaneous species-specific home cage behaviours, and analysed the diurnal variation in these behaviours in inbred mice. Analysis of 18-hour continuous video recordings showed clear alterations in spontaneous home cage behaviours; alterations were at a moderate level after anaesthesia and marked after surgery. Self-grooming, resting and locomotion were the most important behaviours for group separation. Analysis of the temporal distribution of behaviours showed that resting behaviour was altered relative to its normal circadian rhythm as it was decreased in the light phase and increased in the dark phase. Also, locomotion was decreased in the dark phase at 12 to 18 hours after surgery and anaesthesia. In contrast, self-grooming increased independently of circadian rhythm until 18 hours after surgery and anaesthesia. After surgery, there was no significant difference in behaviours found regardless of whether animals were treated with carprofen or not. In conclusion, spontaneous home cage behaviours like resting, locomotion and self-grooming were altered to a moderate degree for at least 18 hours after inhalation anaesthesia and to a high degree after surgery. The efficacy of post-operative pain treatment might not always be detectable by observing only alterations in normal home cage behaviours. The impact of anaesthesia and surgery might thus be considered when interpreting research data, as well as for assessing animal well-being or confirming pain relief measures.

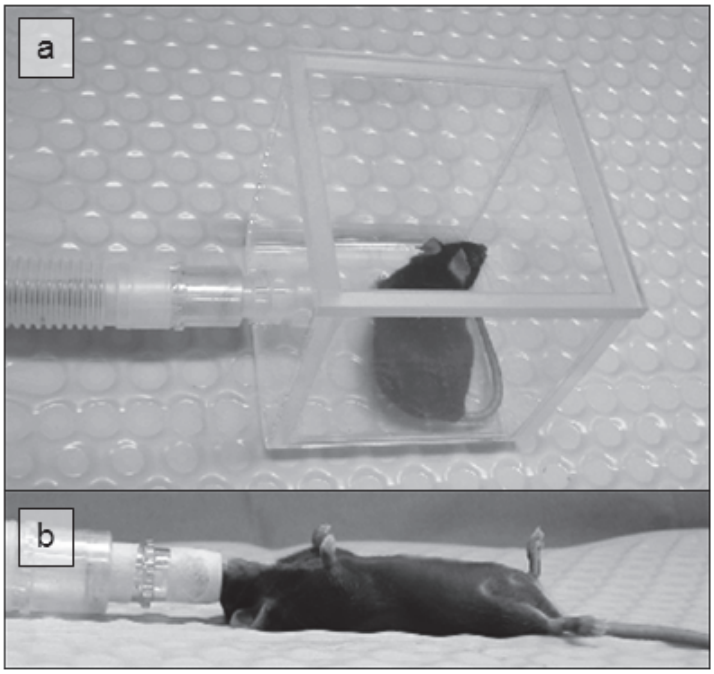


Figure 6: *Inhalation anaesthesia was induced in an induction chamber (a) and provided via a nose mask (b) during experimental surgery.*

Chapter 2 Burrowing behaviour as an indicator of post-laparotomy pain in mice

This study investigated the use of burrowing performance as a potential measure of mild-to-moderate post-operative pain in laboratory mice. The burrowing test is based on the species-typical behaviour of mice to spontaneously displace items from tubes within their home cage; this behaviour has been used as a tool to provoke and determine burrowing behaviour under experimental conditions in models of prion disease (87), brain lesions (88), systemic inflammation (89), and Alzheimer's disease (90). In our study the influence of minor surgery on burrowing was analysed in the commonly used C57BL/6J mice of both sexes, using a modified rodent burrowing test within the animal's home cage. Mice underwent a minor, one-sided laparotomy—a frequent procedure in the generation of genetically modified animals—under inhalation anaesthesia (sevoflurane), with or without pain treatment (5 mg/kg body weight of the non-steroidal anti-inflammatory drug Carprofen). Control animals received anaesthesia/analgesia only. A standard, opaque plastic water bottle filled with food pellets identical to those of the animal's normal diet was provided for burrowing (figure 7), and an additional empty bottle was provided to serve as an alternative shelter for the animal. We measured the latency to the displacement of food pellets (burrowing) from the burrowing apparatus and the duration of burrowing using infrared sensitive cameras to record animals for 24 hours in the absence of a human observer, and measured the weight of removed material after two hours. Latency to burrow was the most valid parameter and might resemble a measure of motivation (91). Almost all (98%) healthy mice burrowed (mean latency 1.3 h). After minor surgery with anaesthesia but no pain treatment, latency of burrowing was significantly prolonged (mean Δ latency 10 h). Analgesic treatment with Carprofen decreased the latency of burrowing after surgery (mean Δ latency 5.5 h) to the level found in mice that had been anesthetized only (mean Δ latency 5.4 h) or had received anaesthesia and analgesia (mean Δ latency 4.6 h). Thus, a significant prolongation in burrowing latency

seems to correlate with post-surgical pain and distress. Moreover analgesia during surgery was associated with a significantly earlier onset of burrowing compared to surgery without pain treatment.

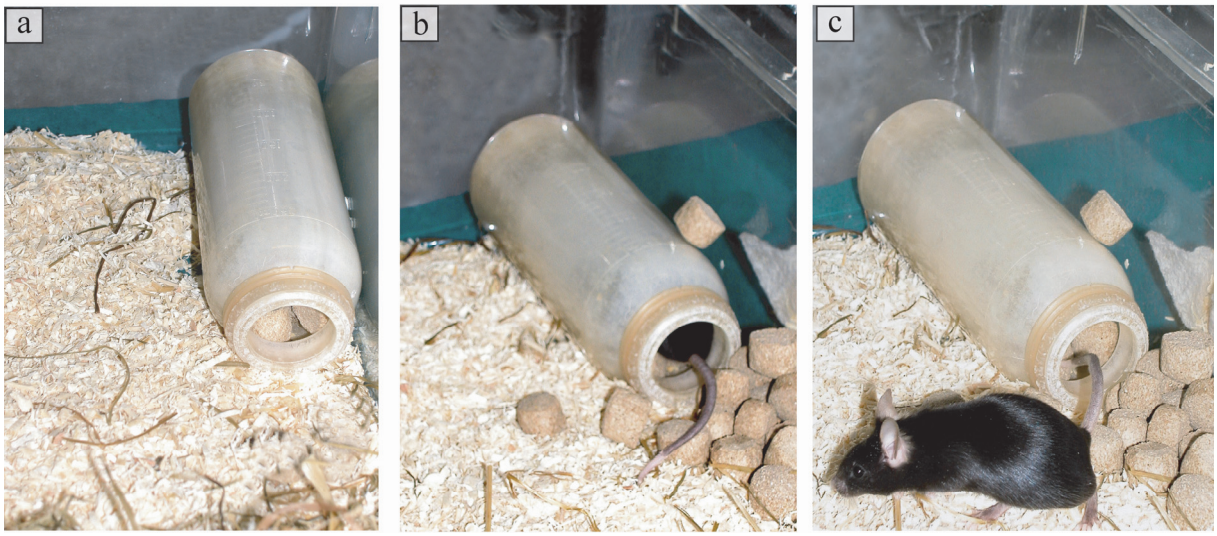


Figure 7: *Burrowing: a) material-filled tube in cage, b) mouse inside and c) mouse in front of tube; removed material in front of tube.*

Chapter 3 Burrowing is a sensitive behavioural assay for monitoring general well-being during DSS colitis in laboratory mice

To test whether changes in burrowing performance are also valid indicators for other kinds of pain and distress, we tested the burrowing test in a model of chronic inflammatory bowel disease (IBD). An impaired intestinal epithelial barrier is thought to be a major factor in the pathogenesis of human IBD. IBD is frequently investigated by inducing a damaged barrier in murine models of colitis. This can be done by feeding mice with dextran sulfate sodium (DSS) polymers in the drinking water. Refinement measures should focus on alleviating unnecessary suffering during this probably painful condition. Appropriate parameters are needed to decide when to terminate the experiments. Our aim was to investigate whether changes in burrowing behaviour is a sensitive measure of animal welfare in murine models of colitis. Acute colitis was induced in 23 C57BL/6 mice with 2% DSS over nine days. The presence of intestinal inflammation upon acute DSS-induced colitis was confirmed by a decrease in body weight, colon length and an increase in murine endoscopic index of colitis severity (MEICS), histological score and spleen weight in the group receiving DSS as compared to the control group. Onset of intestinal inflammation correlated with a significant decrease in burrowing behaviour ($p < 0.05$). Altered adrenal gland histology indicated stress upon acute colitis. Our findings provide evidence that changes in spontaneous burrowing behaviour correlate with the onset of inflammation in acute DSS-induced colitis. We suggest the burrowing test testing as a promising and simple experimental setup to assess impact of colitis on animal well-being.

Chapter 4 Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring

In a previous study we demonstrated a general correlation of post-operative pain and nest building performance in laboratory mice (54). However, a standardised protocol for the assessment of pain using nest building performance has so far not been developed.

Here, we investigated nest building under various conditions, and scored nest complexity to assess postsurgical pain. Mice of both sexes, different strains [C57BL/6J, DBA/2J, and B6D2-Tg(PrmSMalphaActin) V5rCLR-25], and kept under different housing conditions, showed no differences in their latency to use the offered nest material. Healthy female C57BL/6J mice were engaged 4.3% of the day with nest building and showed three peaks of this behaviour: in the beginning and middle of the light phase, and in the second half of the dark phase. We used one cotton nestlet as nest building material (figure 8) and a nest complexity rating scale to standardise nest scoring for the assessment of post-operative pain (score 0 = no nest building activity, to score 5 = complex nest) in female C57BL/6J mice. As mice tend to destroy and rebuild their nests in a circadian rhythm, successful assessment of nest building performance depends on the time at which the nest is observed. Therefore we analysed the normal 24-h nest building rhythm in healthy mice to determine a suitable time to score nest complexity. For assessment of postsurgical pain, female C57BL/6J mice underwent a sham embryo transfer with or without different doses of the analgesic carprofen or control treatment. Nest complexity scoring at 9 h after the experimental treatments (i.e. at the end of the light phase) resulted in less than 10% of animals with noticeably manipulated nest material (nestlet) after surgery and more than 75% of healthy mice having built identifiable-to-complex nests or had noticeably manipulated nestlets, while animals after anaesthesia only showed intermediate nest complexity. Carprofen analgesia resulted in no (5 mg/kg) or only slight (50 mg/kg) improvement of nest complexity after surgery. Thus, nest complexity scoring can be incorporated into daily laboratory routine and can be used in mice as a sensitive tool for detecting reduced well-being and general condition, but probably not for determining the efficacy of pain treatment.

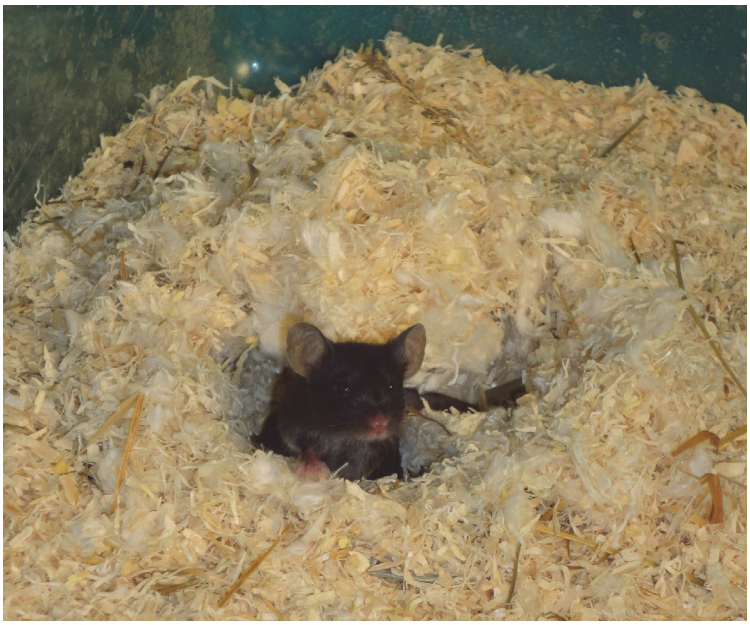


Figure 8: *Female C57BL/6J mouse in nest made of one cotton nestlet.*

Influence of animal housing on welfare and coping

The environment of an animal consists of different stimuli including social environments, human contact and the physical environment of the animal's cage and its contents (85). Housing and husbandry have a major impact on the laboratory animal throughout its life (92). Additionally it has been demonstrated that animal husbandry conditions can influence pain sensitivity or the way animals react to stressful procedures (34, 93). The transportation of laboratory mice to a new clean cage as well as short-term individual housing are standard

procedures after surgery but may increase vulnerability to surgical stress or interfere with postsurgical recovery. Therefore we monitored the immediate post-surgical period to assess pain and general impairment under different housing conditions.

Chapter 5 Individual housing of female mice: influence on post-surgical behaviour and recovery

Individual housing of laboratory mice may increase vulnerability to surgical stress, and interfere with postsurgical recovery. To analyse the effect of housing conditions on recovery, pair- and single-housed female C57BL/6J mice underwent a minor laparotomy +/- analgesia, anaesthesia only or no treatment. Animals were monitored using non-invasive methods during the immediate post-surgical period to assess pain and general impairment. While no appearance or posture abnormalities were observed post-experiment, home cage behaviours were affected distinctly. Discriminant analysis identified self-grooming, locomotion, climbing and resting as mainly responsible for experimental group separation. Behavioural rhythmicity was disrupted, and behaviours related to well-being, such as nest building, climbing and burrowing, decreased. Behavioural pain signs (e.g. press) increased. Most behavioural alterations showed a gradation between treatments, e.g. burrowing latency ranged from an intermediate level following anaesthesia only and surgery with analgesia, to pronounced prolongation after surgery without analgesia. Significantly lower burrowing performance after surgery without analgesia in individually housed animals indicates better recovery in pairs. Social interaction in pairs—an important component of normal behaviour (64%) and a potential indicator for direct social support—was nearly absent (0.3-0.5%). While anaesthesia and surgery resulted in clear changes in behaviour, differences between housing conditions were minor. Hence, despite a tendency towards better recovery in pairs, we found no distinct negative effect of individual housing. In conclusion, both housing conditions are acceptable during the period immediately following minor surgery, though social housing is always preferable in female mice.

Chapter 6 Housing of female mice in a new environment and its influence on post-surgical behaviour and recovery

The transportation of mice to a new clean cage after surgery is a standard procedure but might have detrimental effects during the critical post-surgical recovery phase. To analyse the effect of post-surgical housing, female C57BL/6J mice housed in their familiar home cage or in a new environment after minor surgery +/- analgesia, anaesthesia only or no treatment were monitored using non-invasive methods during the immediate post-surgical period to assess pain and general impairment. Behavioural investigations and burrowing test revealed no significant differences between housing conditions in untreated mice. While no appearance or posture abnormalities were observed post-experiment, home cage behaviours were affected distinctly. Behavioural rhythmicity was disrupted, and behaviours related to well-being, such as nest building and burrowing performance were shown to be reduced compared to untreated mice. Burrowing latency ranged from an intermediate level following anaesthesia only and surgery with analgesia, to pronounced prolongation after surgery without analgesia in animals housed in their home cage, while burrowing latency in animals in new cages was prolonged dramatically after all experimental treatments. General activity and climbing behaviour were high in new cages after treatment, leading to significant interactions between housing and treatment conditions ($p = 0.006$; $p = 0.014$). Such behavioural differences in animals housed in a new environment compared to animals housed in their familiar environment might be interpreted as signs of reduced well-being, agitation and restlessness in the new cages and may hint that animals cope better with surgical stress when

housed in their familiar environment. Post-surgical transport to a new and clean cage might therefore be an additional stressor after an exhausting event and may affect recovery.

CHAPTERS

Chapter 1: Impact of inhalation anaesthesia, surgery and analgesic treatment on home cage behaviour in laboratory mice

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Impact of inhalation anaesthesia, surgery and analgesic treatment on home cage behaviour in laboratory mice

Key words: mice, anaesthesia, surgery, behaviour, refinement, animal well-being

Abstract

Anaesthesia and analgesia are used frequently in laboratory routine to ensure animal welfare and good scientific outcomes in experiments that may elicit pain or require immobilisation of the animal. However, there is concern regarding the effect of these procedures on animal behaviour in subsequent experiments. Our study determined the impact of short inhalation anaesthesia (sevoflurane, 15 min, 4.9%) and minor surgery with or without pain treatment (carprofen, 5 mg/kg, bid) on spontaneous species-specific home cage behaviours in inbred mice. Analysis of 18-hour continuous video recordings showed clear post-procedural changes in spontaneous home cage behaviours, with changes of a moderate level after anaesthesia being marked after surgery. Self-grooming, resting and locomotion were the most important behaviours for group separation. Analysis of the temporal distribution of behavioural changes revealed that resting behaviour was altered contradictory to its circadian rhythm as it was decreased in the light phase and increased in the dark phase. Also, locomotion was decreased in the dark phase at 12 to 18 hours after surgery and anaesthesia. In contrast, self-grooming was increased independently of circadian rhythm, being increased for up to 18 hours after surgery and anaesthesia. Following surgery, there was no significant difference in duration of behaviours between animals that were treated with carprofen or left without pain relief. In conclusion, it can be assumed that the changes observed in home cage behaviours hint at reduced animal well-being. However, pain or the efficacy of post-operative pain treatment could not be discriminated reliably from the impact of the surgical procedure including inhalation anaesthesia by observing animals' home cage behaviour. However for the interpretation of behavioural research data, the distinct impact of anaesthesia, surgery, pain treatment and other experimental procedures has to be considered. Our results highlight the requirement for knowledge of species-specific circadian rhythms of behaviours as well as the importance of determining the appropriate time of day for behavioural and welfare assessment.

Introduction

Laboratory mice are currently the most widely used animal species in biomedical research. Due to their manageable size, a wealth of inbred or genetically modified strains and plenitude of established experimental protocols, mice are used increasingly in complex investigations. These often require induction of general anaesthesia for performing special diagnostic manipulations (e.g., imaging procedures, endoscopy, blood collection), or surgical procedures that in turn require peri- and/or post-operative pain treatment. Analgesic treatment would seem necessary after invasive procedures like laparotomy, but has been omitted frequently in the past (1, 2). Reasons may vary from concern that analgesic use may compromise the data obtained from the proven model to the difficulties of detecting and interpreting signs of pain after minor surgery in mice (e.g. (2)).

Recently, it has become apparent that the physiological and behavioural changes induced by minor or moderate surgery can last up to 24 - 48 hours (3, 4). Moreover, it has been shown that changes induced by anaesthesia, and possibly also by treatment-related procedures (e.g. handling, transport to operating theatre etc.), may effect physiology and animal well-being for several hours (5, 6). Thus it can be assumed that, in some situations, the

effects of anaesthesia may overlap and to some extent mask the post-operative effects of pain. In addition, although the impact of volatile anaesthetic agents on learning, memory, solving of spatial tasks and activity has been studied recently (7-9), the effects of anaesthesia, as an integral part of standard surgical procedures, on spontaneous home cage behaviours have been described only rarely (10). Since anaesthesia, in particular inhalation anaesthesia, is used increasingly in the laboratory routine of biomedical research, questions regarding the duration and persistence of long-lasting anaesthetic or procedural effects come into focus (1).

There is concern regarding not only animal welfare but also the reliability of data obtained from research using animals that have undergone procedures that may elicit pain and/or involve analgesic and/or anaesthetic treatment. This study aimed to determine the effects of minor surgery with or without pain treatment, as well as the impact of standard, short inhalation anaesthesia alone on spontaneous and species-specific home cage behaviours in two common inbred mice strains. To this end, the overall temporal distribution of the animals' natural behaviours was investigated according to their circadian rhythmicity in order to identify whether specific behaviours are altered significantly after surgery or inhalation anaesthesia.

Methods

Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to European Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 2011).

Animals

A total of 64 C57BL/6J and DBA/2J mice of both sexes were obtained from our in-house breeding facility at the age of 6–8 weeks. The health status of the animals was monitored by a health surveillance program according to FELASA guidelines throughout the experiments. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations (11), except for Helicobacter species.

All animals were housed in groups of three to eight animals of the same sex for at least 3 weeks prior to testing in our animal room. Animals were kept in type 3 clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding and two nestlets™ (each 5 cm × 5 cm) consisting of cotton fibres (Indulab AG, Gams, Switzerland) as nesting material. Additionally, animals were provided with a transparent plastic shelter (Mouse house™, Indulab, Gams, Switzerland). They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum (provided in the food hopper continuously throughout the entire duration of the experiment) and had unrestricted access to sterilised drinking water. The light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 Lux in the cage). The temperature was 21 ± 1°C, with a relative humidity of 55 ± 10%, and the air pressure was controlled at 50 Pa with 15 complete changes of filtered air per hour (HEPA H 14 filter). The animal room was insulated to prevent electronic and other noise. Disturbances, e.g., visitors or unrelated experimental procedures, were not allowed.

Experimental groups

In order to distinguish between the effects of inhalation anaesthesia and surgery with or without analgesic treatment, 64 animals (4 per sex and strain) were allocated randomly to one of four treatment groups: 1) the "anaesthesia" group (A), which received inhalation anaesthesia only; 2) the "surgery + anaesthesia + analgesia" group (S+), which underwent inhalation anaesthesia and minor surgery with analgesic treatment; 3) the "surgery + anaesthesia" group (S-), which underwent anaesthesia and minor surgery without analgesic treatment; and 4) a control group, which received no treatment (C).

Experimental treatments and data recording

For acclimatisation, animals were housed individually for 3 days as described in detail above. The experimental treatment began 2 hours before light phase with a subcutaneous injection of 2 µl/g body weight of phosphate buffered saline (PBS) for groups S and A. In the S+ group, 5 mg/kg body weight of the non-steroidal anti-inflammatory drug (NSAID) carprofen (Rimadyl™, Pfizer Inc., NY, USA) was diluted in PBS and injected as 2 µl/g body weight. Forty-five minutes later, the animals were transferred in individual transport cages to the operating theatre, which was located nearby. Mice were anesthetised with sevoflurane (Sevorane™, Abbott, Baar, Switzerland) as a mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen (100%) was used as carrier gas. After induction of anaesthesia in a Perspex induction chamber (8% sevoflurane, 600 ml/min gas flow) for 2 minutes, animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 39°± 1°C to ensure constant body temperature, and anaesthesia was maintained via a nose mask (4.9% sevoflurane, 600 ml/min oxygen flow). The fur was clipped and the operating field disinfected with ethanol in all animals. Male and female mice of both surgery groups underwent a one-side sham vasectomy or a one-side sham embryo transfer, respectively. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl™, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany) and the skin was closed using skin staples (Precise™, 3M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in both surgery groups. Anaesthesia lasted 14–16 min in all 3 treatment groups. Animals were allowed to recover for 15–20 min on the warming mat before being transferred back to the animal room for subsequent video recording. All experimental and control recordings began at the start of the light phase shortly after returning the mouse from its transport cage to its home cage.

Behavioural analysis

Behaviour was recorded digitally in the absence of a human observer with infrared sensitive cameras. The recorded material (18 hours of continuous footage) was subsequently analysed by trained and trial-blinded personnel using ObserverXT™ software (Noldus, Wageningen, Netherlands). The duration of locomotion, self-grooming, resting, eating, drinking and nest building behaviour was measured (Table 1, (12)).

home cage behaviours	
resting	motionless state, no activity (sitting or lying flat, sometimes with the eyes closed or nearly closed, includes sleeping)
locomotion	oriented movement including walking, running, jumping and grid climbing
self grooming	wiping, licking and nibbling the fur with forepaws and tongue, but also scratching and claw cleaning
eating	consumption of food
drinking	consumption of water from the water bottle e
nest building	carrying and shredding of the nestlet, arrangement of cotton fibres, creation of a nest

Table 1: *Ethogram of home cage behaviours according to Van Oortmerssen (1970).*

Data were initially summed for the whole 18-hour period. In order to determine the temporal distribution of behavioural changes, the 18 hours were divided into three consecutive 6-hour periods according to the light-dark cycle in the animal room. Data were summed and analysed for the following time frames: 0–6 hours (light phase), 6–12 hours (light phase), and 12–18 hours (dark phase).

Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (IBM, Armonk, USA). All data were tested for normal distribution and homogeneity of variance. If necessary, data were log (X+1) transformed to meet the assumptions of statistical tests.

No significant effect of animal gender was detected with any of the measures. Therefore, a combined data set of males and females was used.

Mean and standard deviation (SD) of total durations of home cage behaviours were calculated.

Discriminant analysis was used to determine the effects of surgery, anaesthesia and analgesic treatment on home cage behaviour; behaviours mainly responsible for group separation were determined. The total durations of determined behaviours were further analysed using a univariate general linear model (GLM) with experimental group as a fixed factor. Post hoc tests (Bonferroni) were used for comparisons between experimental groups.

Significance for all statistical tests was established at $p \leq 0.05$.

Results

Contribution to group separation was analysed with discriminant analysis of the summed data, initially for the whole 18 hours observation period and subsequently for the three 6-hour periods; 0–6h; 6–12h; 12–18h (Figure 1).

Figure 1

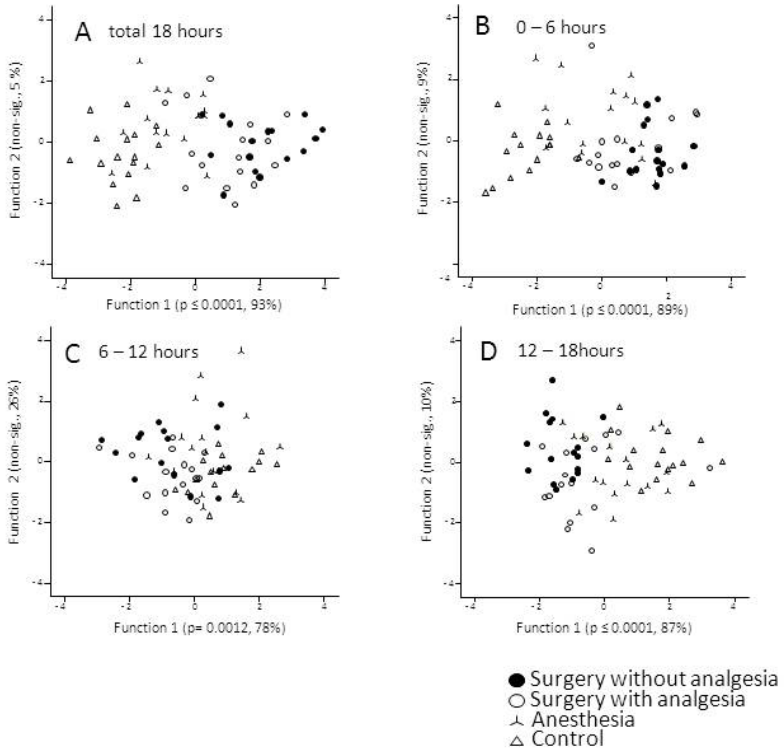

Figure 1: *Scatter plot of discriminant scores assigned to individual mice. The significance of each function in separating groups, and their percentage contribution to between-group variance are shown on each axis. A) 18h observation period. Duration of self grooming and locomotion behaviour contributed most to group separation. B) During the first observation sequence (0-6h post treatment) locomotion and self-grooming behaviour were mainly responsible for group separation. C) During 6–12h post treatment, duration of self-grooming behaviour was mainly responsible for group separation. D) Locomotion, self-grooming and resting contributed significantly to group separation during the 12–18h post-treatment observation period.*

Behaviours that contributed most to group separations were locomotion, self-grooming and resting. These behaviours also represented the largest part of the overall behavioural time-budgets, by far. Based on this observation, only the results for locomotion, self-grooming and resting behaviour were analysed with a GLM and presented in further detail (Figure 2).

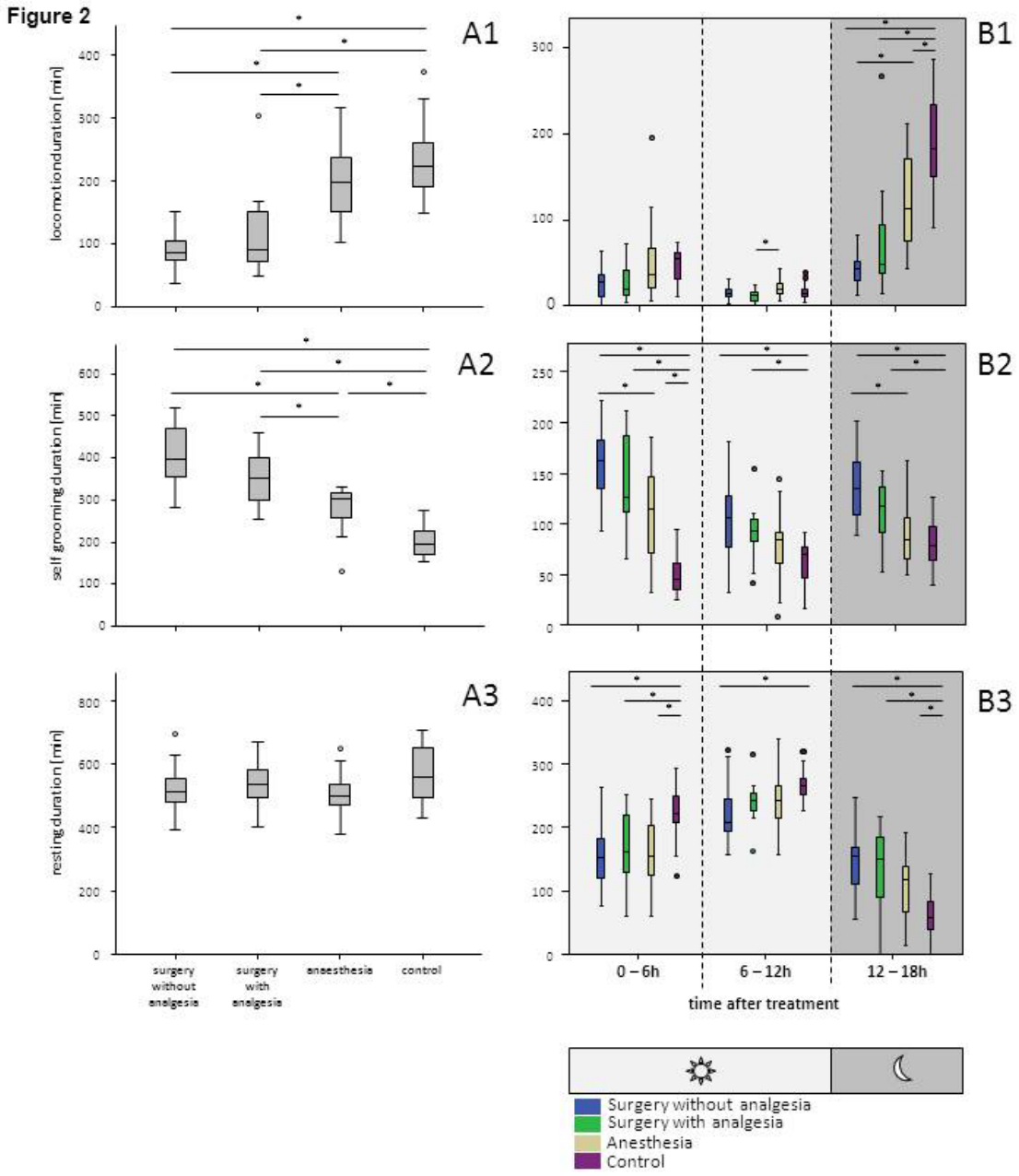

Figure 2: *Effects of anaesthesia and surgery with or without analgesic treatment on duration of 3 spontaneous home-cage behaviours compared to control values. * Significant ($p \leq 0.05$) differences between experimental groups.* ***A)*** *Total duration of locomotion (A1) was decreased post experiment, self-grooming (A2) increased, and resting (A3) behaviour remained unchanged when the whole 18h observation period was analysed.* ***B)*** *Temporal distribution of behavioural effects became apparent when dividing observations into 3 consecutive 6-hour sequences. Duration of locomotion behaviour (B1) was unchanged during the lights-on period but shortened dramatically during the dark period. The increase in duration of self-grooming (B2) was distributed equally in all time sequences, whereas resting (B3) was decreased during the first (0–6h) and increased during the last (12–18h) sequence.*

Effects of treatment on analysed behaviours: total 18-hour observations

During the total 18-hour observation period, the control group (C) (230 min +/- 60) and the anaesthesia group (A) (197 min +/- 60) displayed significantly ($p \leq 0.0001$, each comparison) longer durations of locomotion compared to animals that underwent surgery with (S+) (112 min +/- 63) and without (S-) (89 min +/- 30) pain treatment (Figure 2A 1).

Self grooming behaviour was prolonged significantly after all experimental procedures compared with the untreated C group (199 min +/- 37) ranging from a high level in group S (404 min +/- 71; $p \leq 0.0001$) to an intermediate level in group S+ (351 min +/- 62; $p \leq 0.0001$), with the shortest durations in group A (278 min +/- 54; $p = 0.002$). Additionally, significant differences between the anaesthesia and surgery groups were seen: S- ($p \leq 0.0001$) and S+ ($p = 0.004$) (Figure 2A 2).
No significant differences in resting durations were observed between any treatments: C (568 min +/- 83), S- (523 min +/- 71), S+ (539 min +/- 71) and A (507 min +/- 67) (Figure 2A 3).

Effects of treatment on analysed behaviours: 6-hour observations
By dividing the observations into 6-hour sequences, circadian differences in the effects became apparent (Figure 2B 1-3).
During the first 6h observation period (0–6h), locomotion durations did not show any significant differences between groups S- (26.7 min +/- 18.7) S+ (26.8 min +/- 19.1), A (51.7 min +/- 48.1) and C (47.9 min +/- 19.9). Between 6 and 12 hours post treatment, groups S- (15.9 min +/- 8.4), S+ (11.8 min +/- 7.2) and A (20.9 min +/- 10.5) displayed no significant differences when compared to group C (17.2 min +/- 9.7), while locomotion duration of S+ was significantly shorter than that of group A ($p = 0.036$). In the last observation period (12–18h), corresponding to the first 6 hours of dark phase, all groups showed significantly shorter durations of locomotion compared to the untreated group C (185.7 min +/- 52): S- group (46.7 min +/- 28 ; $p \leq 0.0001$), S+ (73 min +/- 63; $p \leq 0.0001$) and A (121.6 min +/- 57; $p = 0.006$). Further, duration of locomotion was significantly shorter ($p = 0.001$) in group S- than in group A.

Total duration of self grooming during the first 6h observation period (0–6h) was significantly longer in experimental groups S- (162 min +/- 33; $p \leq 0.0001$), S+ (141 min +/- 45; $p \leq 0.0001$) and A (111 min +/- 43; $p \leq 0.0001$) compared to the control group (50 min +/- 20). Additionally, there was a significant difference between groups S- and A ($p = 0.001$). A comparable tendency was seen in the second time period (6–12h). Animals that underwent surgery groomed themselves for significantly longer in groups S- (106.4 min +/- 43; $p = 0.004$) and S+ (96.3 min +/- 33; $p = 0.042$), compared to group C (62 min +/- 22), while in group A self-grooming was prolonged only insignificantly (79.4 min +/- 34, n.s.). In the last observation period (12–18h), animals that received surgery without pain treatment (S-) spent the most time grooming (136.2 min +/- 33; $p \leq 0.0001$) followed by animals that received surgery with pain treatment S+ (113.3 min +/- 27; $p = 0.014$). Compared to the untreated group C, differences were significant (80 min +/- 25). Animals that received anaesthesia only (A) (91.3 min +/- 30) showed significantly shorter grooming durations compared to group S- ($p \leq 0.0001$).

Animals that underwent anaesthesia or surgery spent significantly less time resting in the first observation period (0–6h) compared to the untreated group C (224 min +/- 44): S- (154 min +/- 47; $p = 0.002$), S+ (165 min +/- 61; $p = 0.013$) and group A (160 min +/- 55; $p = 0.006$).
In the second observation period (6–12h), resting duration was significantly shorter in group S- (220.9 min +/- 49; $p = 0.011$) compared to the untreated group C (267.8 min +/- 27), with somewhat shorter resting duration in groups S+ (237.4 min +/- 37) and A (239.7 min +/- 45). Animals that underwent surgery and anaesthesia spent significantly more time resting compared to untreated controls (60.1 min +/- 35) in the last observation period (12–18h): S- (148.1 min +/- 49; $p \leq 0.0001$), S+ (137 min +/- 59; $p \leq 0.0001$) and A (108.7 min +/- 50; $p = 0.045$).

Discussion

To assess the impact of inhalation anaesthesia and surgery with or without pain treatment in mice, we used non-invasive behavioural observations that can be applied in the animals' home cage without disturbing the animal or provoking additional stress. Using this system we were able to analyse each animal's behaviour continuously for 18 hours following experimental treatments. In contrast to most physiological and clinical parameters, behaviour can be observed easily in a non-invasive manner and can provide a sensitive correlate of the internal state of an animal. Alterations in the frequency of, or in the latency to display, spontaneous and species-specific behaviours (e.g., rearing, sniffing, walking or burrowing behaviour) (13, 14), as well as the quality of nest construction and structuring of cage territory (4, 5) are recent examples of such behavioural indicators.

The results of this study showed that minor surgery with short inhalation anaesthesia, either with or without pain treatment, induced alterations in spontaneous home-cage behaviours such as self-grooming, resting and locomotion. These changes persisted for up to 18 hours.

When interpreting the data summed over the whole 18-hour observation period, we found that locomotion and self-grooming behaviours were most affected by the experimental procedures. After surgery, animals displayed a marked decrease in locomotion and a strong increase in self-grooming. Self-grooming showed a clear stepwise increase from baseline over anaesthesia only, to surgery with pain treatment, to surgery without pain treatment. Differences in self-grooming between groups were significant except for the difference between surgery with pain treatment and surgery without pain treatment. In contrast to locomotion and self-grooming, there was no effect on the overall duration of resting behaviour if the 18-hour post treatment period was observed as a whole.

Pain treatment with carprofen had no statistically significant effect on alterations of spontaneous home cage behaviours. However, animals that received pain treatment during surgery readily assumed intermediate levels of locomotion and self-grooming compared to the group in which pain treatment was not administered during surgery and the group that underwent anaesthesia only. Therefore, it could be speculated that some, but not complete, amelioration of post-operative pain is achieved by administering the non-steroidal anti-inflammatory drug (NSAID) carprofen at a dosage of 5 mg/kg body weight. However, previous studies using physiological investigations and behavioural testing demonstrated that carprofen provided sufficient relief from post-operative pain (4, 14). Thus, it might be that the alterations of spontaneous home cage behaviours analysed in this study are not ideal parameters for estimating pain alleviation by NSAIDs.

When dividing the observation period into 3 consecutive 6-hour-long time segments according to the light cycle in the animal room, circadian-dependent display patterns could be observed. For the first 12 hours after treatment—corresponding to the complete light phase in the animal room—all animals displayed general low levels of locomotor activity, and no difference could be observed between treated and untreated groups regarding this behaviour. However, in post-treatment hours 12–18 (first 6 hours of the dark phase) locomotion of both surgery groups was reduced by 75% and that of the anaesthesia group by 35% compared to that of the control group. In contrast, the duration of self-grooming behaviour was influenced strongly by treatment in all 3 time segments, showing a marked increase in treated animals as compared to the untreated control group. Remarkably, the effects on duration of self grooming seemed not to be influenced by the light-dark cycle in the animal room. Furthermore, the duration of resting behaviour, which displayed no differences between groups in the summed 18-hour analysis period, showed clear and gradual treatment-related effects when analysed according to the time progression, i.e. in separate 6-hour periods. Effects were most significant during the first 6 hours after treatment, when resting decreased, and at 12–18 hours after treatment, when it increased markedly in comparison to control animals.

In recent years, volatile anaesthetics (e.g., sevoflurane, isoflurane) have been used increasingly in laboratory animal practice due to their safety and association with rapid recovery. Further, inhalation anaesthesia is used in diverse procedures, including neurobiology research in which animals are studied subsequently in behavioural tests and where their performance may be influenced by the persistent effects of anaesthetic drugs (7). Previous studies have demonstrated that inhalation anaesthesia can induce changes in heart rate, core body temperature and faecal corticosterone levels that last for several hours after treatment (6, 15). In this study, we have shown that non-painful, short (15 minutes) sevoflurane inhalation anaesthesia can cause post-anaesthetic alterations in locomotion, self-grooming and resting behaviours that are noticeable for up to 18h after treatment. Therefore, for accurate interpretation of behavioural research data the distinct individual impacts of anaesthesia, surgery, pain treatment and other experimental procedures have to be considered. However, as our study protocol did not determine the extent of effects caused by treatment-related actions (like transport, handling etc.), this question requires further investigation.

Studying behaviour in sufficient detail to detect post-surgery-related changes in spontaneous, home-cage based behaviour patterns, and the effects of drugs upon such behaviours, is quite tedious and time consuming, thus studies are often confined to analysing only a limited range of behaviours or performing assessments only over a very short time frame (13). Determining an optimal observation time-point is one of the major difficulties in behavioural research. For example, the display of signs of pain as well as pain tolerance itself is dependent on circadian rhythm, and thus the need to determine the appropriate time of day for observations (3, 16, 17) renders post-operative pain assessment even more challenging in mice. In this study, we were able to show that all 3 behaviours studied in detail (locomotion, self-grooming and resting) displayed different temporal effect patterns. Whereas the effects on self-grooming were distributed evenly over the whole period analysed, locomotion was changed only during ‘dark’ and resting first decreased and then increased markedly in treated groups. We believe that these data suggest strongly that the effects on spontaneous, home-cage based behaviours caused by anaesthesia and minor surgery are not displayed uniformly throughout the day. Our results highlight the requirement for knowledge of species-specific circadian rhythms of behaviours as well as the importance of determining the appropriate time of day for behavioural and welfare assessment.

Conclusion

Spontaneous home cage behaviours, locomotion, self-grooming and resting, were altered for up to 18 hours in all treatment groups, and a graduation between untreated control, anesthesia and surgery groups was found. Short inhalation anesthesia induced moderate changes whereas the impact of surgery was considerable. Thus it can be assumed that the observed changes in home cage behaviors hint at reduced animal well-being. Pain therapy only partially ameliorated the aforementioned effects, leading to the conclusion that either the chosen dosage was too low or that alterations in the spontaneous home cage behaviors analyzed in this study do not allow NSAID efficiency to be estimated reliably.

While self grooming behavior changed post experimentally independently of circadian rhythm, changes in locomotion and resting behavior were distinctly affected by the time of day.

In conclusion, for proper interpretation of behavioural research data, the distinct impacts of anaesthesia, surgery, pain treatment and other experimental procedures have to be considered. Our results highlight the requirement for knowledge of species-specific circadian rhythms of behaviours as well as the importance of determining the appropriate time of day for behavioural and welfare assessment.

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Chapter 2: Burrowing behaviour as an indicator of post-laparotomy pain in mice

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Burrowing behavior as an indicator of post-laparotomy pain in mice

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Detection of persistent pain of a mild-to-moderate degree in laboratory mice is difficult because mice do not show unambiguous symptoms of pain or suffering using standard methods of short-term observational or clinical monitoring. This study investigated the potential use of burrowing performance – a spontaneous and highly motivated behavior – as a measure of post-operative pain in laboratory mice. The influence of minor surgery on burrowing was investigated in adult C57BL/6J mice of both genders in a modified rodent burrowing test (displacement of food pellets from a pellet-filled tube) within the animal's home cage. Almost all (98%) healthy mice burrowed (mean latency 1.3 h, SEM 0.5 h). After surgery without pain treatment, latency of burrowing was significantly prolonged (mean Δ latency 10 h). Analgesic treatment using the anti-inflammatory drug carprofen (5 mg/kg bodyweight) decreased latency of burrowing after surgery (mean Δ latency 5.5 h) to the level found in mice that had been anesthetized (mean Δ latency 5.4 h) or had received anesthesia and analgesia (mean Δ latency 4.6 h). Analgesia during surgery was associated with a significantly earlier onset of burrowing compared to surgery without pain treatment. A distinct gradation in burrowing performance was found ranging from the undisturbed pre-operative status to the intermediate level following anesthesia/analgesia and surgery with analgesia, to the pronounced prolongation of latency to burrow after surgery without pain relief. In conclusion, post-surgical impairment of general condition, probably mainly attributable to pain, can be conveniently assessed in laboratory mice on the basis of the burrowing test.

Keywords: post-operative pain, pain assessment, C57BL/6J mice, burrowing, species-typical behavior, analgesia, post-laparotomy pain, post-surgical pain

INTRODUCTION

The detection of pain in laboratory animals is essential for both scientific and ethical reasons. The responsibility to ensure the care and welfare of the millions of animals used annually in biomedical research (Baumans, 2004, 2005) requires the recognition and alleviation of pain caused by animal experiments (Gauthier and Griffin, 2005). Furthermore, methods of gauging pain are needed in basic research focused on the mechanisms and patho-physiology of pain, and in the applied context of searching for new and improved painkillers. Such biomedical research is often conducted in mice – currently the most widely used laboratory animal species due to the availability of various genetically modified mice models (Baumans, 2004, 2005). However, diagnosis of pain in the mouse is challenging because this species does not voluntarily exhibit obvious signs of pain, which can be explained by the fact that, as prey animals, mice try to hide signs of pain, suffering or disability in order to avoid attracting predators (Stasiak et al., 2003; Peterson, 2004; van Sluyters and Obernier, 2004).

Extensive research in this field has led to a multitude of analgesiometric tests and models for the detection of acute pain and the identification of allodynia or hyperalgesia in mice (e.g., tail flick latency test, paw licking response, partial sciatic nerve injury model; Chapman et al., 1985; Le Bars et al., 2001; Mogil, 2009). Nevertheless, the assessment of non-acute or persistent pain, for example from surgery or other tissue trauma, which can last a few hours to days, remains difficult in the mouse (Kohn et al., 2007). Signs and symptoms of prolonged pain stages published in articles and guidelines usually refer to severe pain due to major surgery, highly invasive, or otherwise severely noxious interventions. Obvious symptoms such as sunken flanks, neglected grooming, piloerection, hunched back or immobility are clear evidence of a severely impaired, often moribund, health status in mice. Interventions with a lesser impact seem not to evoke such clearly recognizable changes. For example, after routine surgical interventions like laparotomy, mice are well known not to display any easily observable abnormal appearance, posture, or behavior indicating the presence of pain, thus leading to a demand for the development of standard evaluation methods for mild-to-moderate pain (Hawkins, 2002). As behavior can be observed easily in a non-invasive manner and can provide meaningful cues as to the internal state of an animal, analyzing complex behavioral changes (e.g., nest building behavior) in pain states has frequently been suggested as a promising approach to assess both pain severity and the efficiency of pain management regimes (Jourdan et al., 2001; Mogil and Crager, 2004; Mogil, 2009;

Roughan et al., 2009). Currently, examples of extensive and refined ethological measures in mice after laparotomy in their home cage environment have been introduced. Global changes in the frequency of spontaneous behaviors (e.g., rearing, sniffing, walking; Roughan et al., 2009), and in the quality of nest construction and structuring of territory (home cage/areal; Arras et al., 2007) under post-operative pain have been described. However, while the former method is based on specialized automated software analysis, the latter has not yet been standardized, which hampers translation of both methods to a laboratory routine setting.

A promising and simple experimental setup with which to assess changes in spontaneous burrowing behavior correlating with different impairments in rodents was published by Deacon and co-authors (Deacon et al., 2001; Deacon, 2006). The test is based on the species-typical behavior of mice to spontaneously displace items from tubes within their home cage; this behavior was used as a tool to provoke and determine burrowing behavior under experimental conditions in models of prion disease (Deacon et al., 2001), brain lesions (Deacon et al., 2003), systemic inflammation (Teeling et al., 2007), and Alzheimer's disease (Deacon et al., 2008). In these models, a decrease in burrowing behavior was interpreted to be correlated with chronic neurological or immunological disorders. As burrowing behavior, although highly motivated (Sherwin et al., 2004), is not an essential maintenance behavior like food consumption or sleeping, we suggest that it is sensitive to different, subtle kinds of impact on the animals' general condition and wellbeing. Changes in this species-typical behavior might therefore be useful as an indicator of pain after surgery.

We used a modified version of the burrowing test setting within each animal's home cage to determine the pre- and post-operative burrowing performance of male and female mice. To induce post-operative pain we performed a sham vasectomy or embryo transfer, both including a laparotomy (Martin et al., 2004; Arras et al., 2007; Roughan et al., 2009). To distinguish between the effects of surgery, anesthesia, and analgesic treatment, we tested groups of mice that underwent surgery with or without the analgesic carprofen or procedural control treatments with anesthesia only or anesthesia plus analgesia. The results presented establish the reliability and feasibility of an easy-to-perform burrowing test with the aim of assessing post-operative pain in mice.

MATERIALS AND METHODS

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (Council of Europe nr. 123 Strasbourg 1985) and to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

ANIMALS

The animals were 32 male and 32 female C57BL/6J mice obtained from our in-house breeding facility at the age of 6–8 weeks. All animals were housed in groups of three to eight animals of the same sex for at least 3 weeks prior to testing in our animal room. Animals were kept in type 3 clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding and two nestlets™ (each 5 cm × 5 cm), consisting of cotton fibers (Indulab AG, Gams, Switzerland) as nesting material. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) *ad libitum* (provided in the food hopper continuously throughout the entire duration of the experiment) and had unrestricted access to sterilized drinking water. The light/dark cycle in the room consisted of 12/12 h (lights on 15:00, lights off 03:00) with artificial light (40 Lux in the cage). The temperature was 21 ± 1°C, with a relative humidity of 50 ± 5%, and the air pressure was controlled at 50 Pa with 15 complete changes of filtered air per hour (HEPA H 14 filter). The animal room was insulated to prevent electronic and other noise. Disturbances, e.g., visitors or unrelated experimental procedures, were not allowed.

EXPERIMENTAL SETUP

As a burrowing apparatus, a plastic bottle (standard opaque water bottle, 250 ml, 150 mm length, 55 mm diameter) filled with 138–142 g of food pellets identical to those of the animal's normal diet was used. An additional empty bottle of the same dimensions was provided to serve as a shelter for the animal (**Figure 1**).

EXPERIMENTAL DESIGN

To test the effects of surgery on burrowing behavior, each animal was tested in the burrowing apparatus before (baseline) and after an experimental procedure (experimental). The individual baseline values were used to compensate for inter-individual variation in burrowing behavior. Eight animals of each sex were allocated randomly to one of four treatment groups: (1) the "surgery + anesthesia" group, which underwent anesthesia and minor surgery without analgesic treatment; (2) the "surgery + anesthesia + analgesia" group, which underwent anesthesia and a minor surgery with analgesic treatment, (3) the "anesthesia" group, or (4) the "anesthesia + analgesia" group; groups 3 and 4 being procedural control groups that underwent only anesthesia with (4) or without (3) analgesia (**Figure 2**).

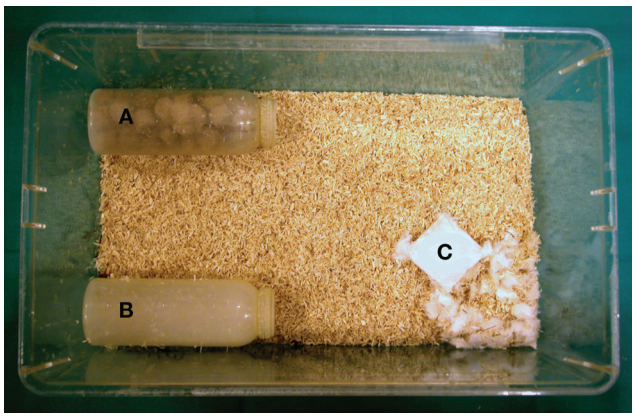

FIGURE 1 | Experimental setup. Burrowing test apparatus **(A)**, shelter **(B)**, and nesting material **(C)** in home cage.

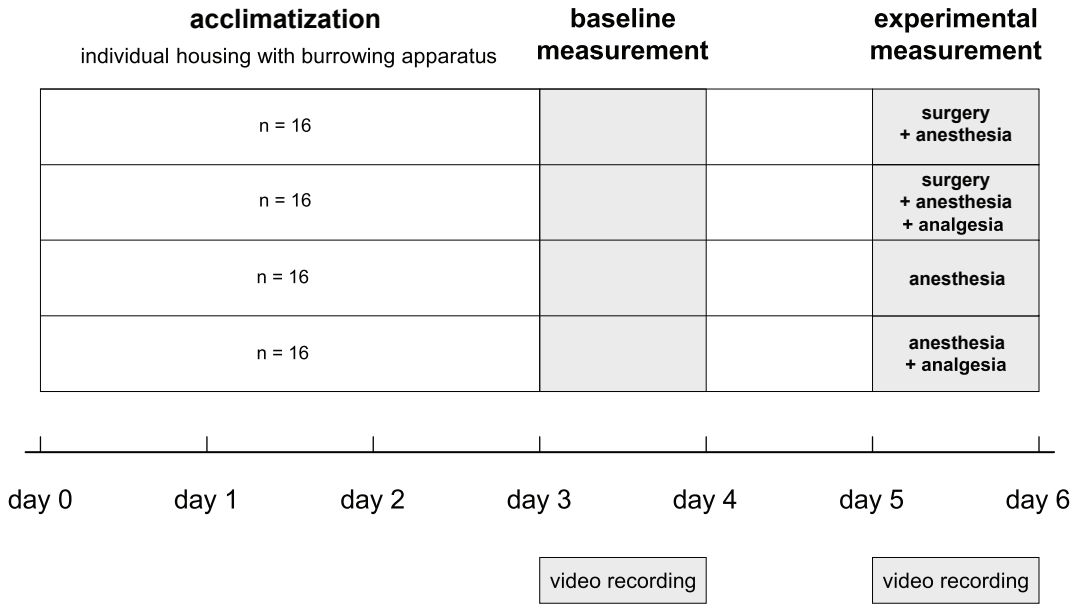

FIGURE 2 | Experimental design and chronological order of experimental procedures and behavioral testing.

ACCLIMATIZATION AND BASELINE MEASUREMENTS

For acclimatization, animals were housed individually for 3 days in a standard cage with food and water *ad libitum* containing the experimental setup as described above (**Figure 1**).

Other than this prior experience with the burrowing apparatus, the animals had no experience with behavioral testing and were not selected for burrowing performance.

Measurements of baseline values of burrowing behavior started at 15:00 hours and were carried out in the animal room. Food pellets removed or eaten by the mice during the preceding days were replaced in the apparatus before testing started. All tests were recorded digitally for 24 h in the absence of a human observer with an infrared-sensitive camera fixed above the cage.

TREATMENTS AND EXPERIMENTAL MEASUREMENTS

Experimental measurements of burrowing behavior were performed 2 days after baseline measurements. The treatment began at 13:00 hours with a subcutaneous injection of 2 µl/g body weight of phosphate buffered saline (PBS) for the surgery + anesthesia and anesthesia groups. In the surgery + anesthesia + analgesia and anesthesia + analgesia groups, 5 mg/kg body weight of the analgesic carprofen (Rimadyl™, Pfizer Inc., NY, USA) was diluted in PBS and injected as 2 µl/g body weight. Forty-five minutes later, the animals were transferred in individual transport cages to the operating theater, which was located nearby. Mice were anesthetized with sevoflurane (Sevorane™, Abbott, Baar, Switzerland) as a mono-anesthesia. The anesthetic gas was provided with a rodent inhalation anesthesia apparatus (Provet, Lyssach, Switzerland); pressurized air was used as carrier gas. After induction of anesthesia in a Perspex induction chamber (8% sevoflurane, 600 ml/min airflow) animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 39°± 1°C to ensure constant body temperature and anesthesia was maintained via nose mask (4.9% sevoflurane, 600 ml/min airflow). The fur was clipped and the operating field disinfected with ethanol in all animals. Male and female mice of both surgery groups underwent a one-side sham vasectomy or a one-side sham embryo transfer, respectively. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl™, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany) and the skin was closed using skin staples (Precise™, 3M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in both surgery groups. Anesthesia lasted 14–16 min in all four treatment groups. Animals were allowed to recover for 15–20 min on the warming mat before they were transferred back to the animal room for subsequent behavioral testing.

The burrowing test began at 15:00 hours by returning the mouse from its transport cage to its home cage containing the refilled test apparatus and by starting the digital video recording.

BEHAVIORAL ANALYSIS

The recorded 24-h video sequences were analyzed using ObserverXT™ software (Noldus, Wageningen, Netherlands). Burrowing was defined as the removal of more than three pellets from the apparatus within 10 s. The latency to onset of burrowing behavior (latency to burrow), and the duration between the start of burrowing and the total emptying of the apparatus (duration of burrowing), were measured in seconds. Additionally, to allow comparison with existing literature, the weight (in grams) of food pellets removed after 2 h of testing was calculated indirectly by counting pellets removed on video files.

CLINICAL INVESTIGATION

Animals were investigated for abnormalities of appearance (e.g., fur, eyes), posture, and movement at the end of the baseline and experimental recordings (15:00 hours at day 4 and day 6) according to a routinely used scoring system (Arras et al., 2007).

STATISTICAL ANALYSIS

All statistical analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

Mean and standard error of the mean (SEM) of latency to burrow, duration of burrowing and weight of removed food pellets were calculated for the baseline measurements and for experimental measurements of the four treatment groups. Additionally, mean, SEM and the 95% confidence interval were calculated for the baseline latency to burrow and the duration of burrowing of all 64 animals.

To consider inter-individual variation the individual baseline values were used to compare experimental latencies, durations and pellet weights between groups. Therefore, mean and SEM were additionally calculated for all groups using the differences (Δ) in latencies, durations and pellet weights between experimental and baseline measurements of each animal.

All data was tested for normal distribution and homogeneity of variance. With the exception of the weight of removed food pellets, all data met the necessary assumptions for parametric analyses.

Absolute and Δ latencies and durations were compared between genders with independent two-sample *t*-tests, with absolute and Δ weight of removed pellets being compared with the Mann–Whitney *U* test.

Dependent *t*-tests for paired samples were used for the comparison of baseline and experimental absolute latencies and durations of each experimental group. To compare baseline and experimental values of weights of removed pellets of each group, the Wilcoxon test for paired data was used.

Experimental absolute and Δ latencies and durations between groups were compared using a one way analysis of variance (ANOVA); *post hoc* testing was conducted with the Tukey test. To compare absolute and Δ weights of removed pellets between groups, the Kruskal–Wallis test was performed.

Kaplan–Meier survival analysis to examine the distribution of time to effect (latency to burrow) for the four different treatments was performed. To test whether the latency to burrow differed statistically between treatment groups, a log rank significance test was performed.

Significance for all statistical tests was established at $p < 0.05$.

RESULTS

No significant effect of the animals' gender was detected with any of the measures. Therefore results are reported for the combined data set of males and females.

BURROWING PERFORMANCE

Figure 3 presents the percentage change in the fraction of mice that started burrowing during baseline and experimental recordings. Out of 64 animals only one male did not burrow, either during the 24 h of baseline recording or during the 24 h of experimental recording. During the 24 h baseline recordings, the majority (89%) of mice started burrowing within the first hour of the test, whereas most mice in all the experimental groups started burrowing considerably later. The fraction of mice that started burrowing increased similarly in the anesthesia, anesthesia + analgesia and surgery + anesthesia + analgesia groups within 12 h of observation. While anesthesia and surgery + analgesia groups reached baseline values after 13 and 16 h of recording, respectively, the anesthesia + analgesia group did not reach baseline values until 23 h. The fraction of mice starting burrowing in the surgery + anesthesia group was distinctly lower and did not reach values of baseline recordings during the 24 h of observation.

BASELINE VS. EXPERIMENTAL VALUES

During baseline measurements, mice started burrowing on average at 1.3 h, SEM 0.5 h (95% confidence interval: 0.32, 2.3 h) and emptied the burrowing apparatus within 0.83 h, SEM

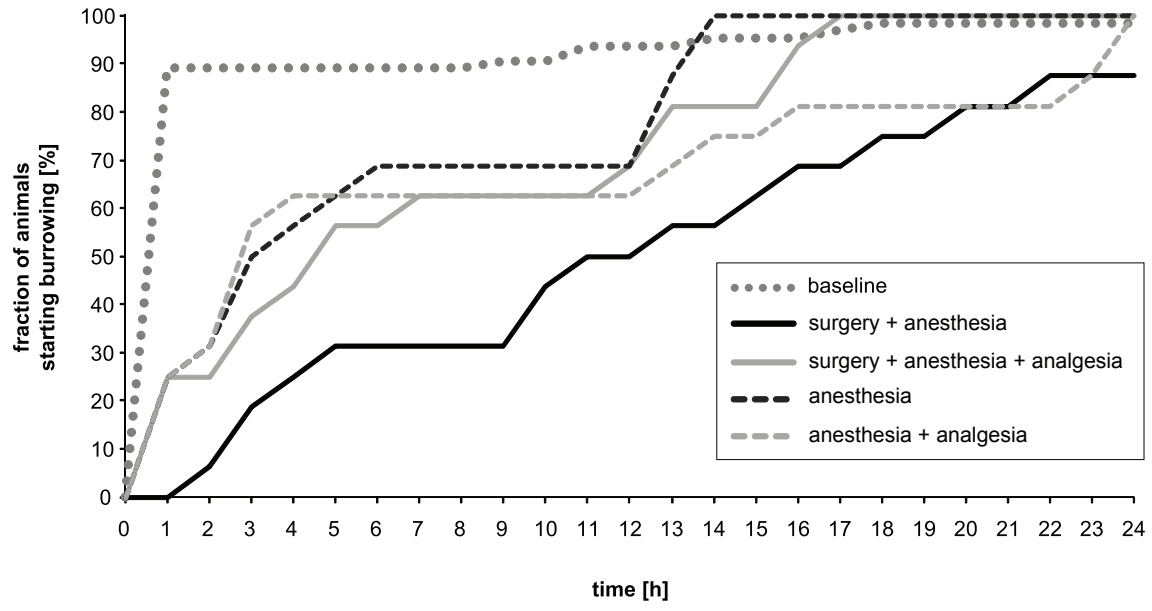

FIGURE 3 | Percentage of mice/hour that started burrowing at baseline vs. experimental conditions. Healthy mice started burrowing immediately, mostly within 1 h (baseline). Between-treatment differences were greatest between hours 7 and 9.

0.33 h (95% confidence interval: 0.17, 1.5 h). All the experimental treatments caused marked changes in the latency to burrow, duration of burrowing and the weight of removed pellets at 2 h.

Compared to the respective baseline values, mice showed significantly longer latencies after experimental treatment in the surgery + anesthesia group, surgery + anesthesia + analgesia group, and anesthesia group, but a tendency toward longer experimental latencies only in the anesthesia + analgesia group (**Table 1**).

Mean of duration of burrowing tended to be higher after all experimental treatments compared to baseline values, but this difference could not be confirmed as statistically significant (**Table 1**).

The amount of pellets removed at 2 h after treatment was significantly less than baseline values in all four groups (**Table 1**).

EFFECTS OF TREATMENT ON BURROWING BEHAVIOR

Comparison of experimental groups revealed distinct differences in the effects of the respective treatments on latency to burrow and amount of pellets removed at 2 h. Surgery without pain relief caused considerably longer absolute and Δ latencies and lower weight of removed pellets than surgery with analgesia, anesthesia, or anesthesia + analgesia but no relevant difference in absolute and Δ duration of burrowing (for detailed results see **Tables 1 and 2**).

No statistically significant difference could be observed either in absolute latency to burrow, duration of burrowing and weights of pellets, Δ latency and Δ duration, while Δ amounts of removed pellets show significant differences (**Table 3**).

The distribution of time to effect, i.e., latency to burrow traced in Kaplan–Meier analysis, exhibited a distinct shift of the surgery + anesthesia group compared to the other three treatment groups (**Figure 4**). The log rank analysis showed a significant association between treatment of groups and onset of burrowing: the surgery + anesthesia group started to burrow significantly later compared to the surgery + anesthesia + analgesia group and the anesthesia group. All other group comparisons showed no significant differences (surgery + anesthesia + analgesia vs. anesthesia: $p = 0.260$, $\chi^2 = 1.270$; surgery + anesthesia + analgesia vs. anesthesia + analgesia: $p = 0.479$, $\chi^2 = 0.502$; anesthesia vs. anesthesia + analgesia: $p = 0.180$, $\chi^2 = 1.798$).

CLINICAL INVESTIGATION

Observations of appearance, posture, and movement revealed no difference between baseline and experimental measures, or between experimental groups.

Table 1 | Absolute values of latency to burrow, duration of burrowing and weight of removed food pellets. Results of paired *t*-test for latency to burrow and duration of burrowing, and Wilcoxon test for removed pellet weights.

			Mean (h)	SEM (h)	*p*-Value	*t*
Latency to burrow	Surgery + anesthesia	Baseline	0.09	0.02	0.0005*	–5.779
		Experimental	10.41	1.79		
	Surgery + anesthesia + analgesia	Baseline	1.58	1.02	0.004*	–3.431
		Experimental	7.09	1.66		
	Anesthesia	Baseline	0.10	0.04	0.001*	–4.07
		Experimental	5.48	1.32		
	Anesthesia + analgesia	Baseline	3.38	0.49	0.081$^{n.s.}$	–1.871
		Experimental	7.90	2.16		
Duration of burrowing	Surgery + anesthesia	Baseline	0.27	0.01	0.193$^{n.s.}$	–1.387
		Experimental	1.89	0.27		
	Surgery + anesthesia + analgesia	Baseline	0.46	0.31	0.148$^{n.s.}$	1.601
		Experimental	1.63	0.38		
	Anesthesia	Baseline	0.45	0.13	0.190$^{n.s.}$	–1.377
		Experimental	1.85	0.23		
	Anesthesia + analgesia	Baseline	0.66	0.08	0.194$^{n.s.}$	–1.405
		Experimental	3.81	0.37		
			Mean (g)	**SEM (g)**	***p*-Value**	***Z*-value**
Weight of removed food pellets at 2 h	Surgery + anesthesia	Baseline	134.1	5.9	0.0005*	–3.556
		Experimental	10.3	9.3		
	Surgery + anesthesia + analgesia	Baseline	134.3	5.7	0.001*	–3.357
		Experimental	35	15.7		
	Anesthesia	Baseline	133.2	5.2	0.001*	–3.438
		Experimental	30.4	13.8		
	Anesthesia + analgesia	Baseline	86.2	15.4	0.004*	–2.852
		Experimental	33	14.1		

** = $p < 0.05$; n.s. = $p > 0.05$.*

Table 2 | Delta (Δ) values of latency to burrow, duration of burrowing, and weight of removed food pellets.

		Mean (h)	SEM (h)
Δ Latency to burrow	Surgery + anesthesia	10.3	1.8
	Surgery + anesthesia + analgesia	5.5	1.6
	Anesthesia	5.4	1.3
	Anesthesia + analgesia	4.6	1.6
Δ Duration of burrowing	Surgery + anesthesia	1.6	0.2
	Surgery + anesthesia + analgesia	1.2	0.5
	Anesthesia	1.4	0.2
	Anesthesia + analgesia	3.3	0.5
		Mean (g)	**SEM (g)**
Δ Weight of removed food pellets at 2 h	Surgery + anesthesia	–123.7	10.6
	Surgery + anesthesia + analgesia	–99.3	15.8
	Anesthesia	–102.8	13.8
	Anesthesia + analgesia	–53.4	16.0

Table 3 | Results of ANOVA for experimental absolute and Δ latencies to burrow and durations of burrowing and of Kruskall–Wallis test for experimental absolute and Δ weights of removed food pellets for all four groups.

Group comparison	*p*-Value	*F*-value
Absolute latency to burrow	$0.283^{n.s.}$	1.301
Absolute duration of burrowing	$0.530^{n.s.}$	0.746
Δ Latency to burrow	$0.139^{n.s.}$	1.906
Δ Duration of burrowing	$0.222^{n.s.}$	1.518
	***p*-Value**	χ^2
Weight of removed food pellets at 2 h	$0.693^{n.s.}$	1.452
Δ Weight of removed food pellets at 2 h	0.005*	12.791

** = $p < 0.05$; n.s. = $p > 0.05$.*

DISCUSSION

The burrowing test (modified from descriptions by Deacon et al., 2001), was investigated for its feasible use in the assessment of post-operative general condition and probably post-operative pain. The test proved easy to perform in an experimental setting within the animal's home cage.

All burrowing parameters analyzed responded sensitively to the different experimental manipulations performed on the animals. Healthy mice of both genders of the common C57BL/6J strain started burrowing (i.e., displaced substantial numbers of food pellets from a tube) in general within 0.3–2.3 h after this pellet-filled tube was provided to them. After surgery without pain treatment, the latency to burrow was significantly prolonged, i.e., when the pellet-filled tube was presented to them in their home cage after surgery mice started burrowing several hours later. With analgesic treatment, the latency to burrow after surgery was only moderately extended to a level similar to that found in the procedural control groups, i.e., in mice anesthetized only or in mice receiving anesthesia and pain treatment only. Thus, surgery with analgesic treatment in mice resulted in a significantly earlier onset of burrowing compared to surgery without pain relief.

Likewise, the weight of removed pellet material in the default time frame of 2 h (referred to by Deacon et al., 2001) was significantly reduced after any of the treatments. Comparing between treatments, the amount of removed pellets was less after surgery than after surgery with analgesia, anesthesia and analgesia, or anesthesia only. The duration of burrowing (i.e., the time required by the animal to empty the tube) was not significantly different between any of the groups although it was prolonged after all experimental treatments compared to the healthy baseline status.

In our laparotomy model, the results of the assessment of burrowing performance were as expected from common sense, as well as from earlier studies on physiology (Arras et al., 2007), pain research (Langford et al., 2010), and from the results of previously published burrowing testing in different kinds of disorders, including pathological processes in the brain such as prion disease, pre-frontal cortex or hippocampal lesions (Deacon et al., 2001, 2002, 2003, 2008; Teeling et al., 2007). Such brain damage can alter measures of burrowing independently of pain. Burrowing, as a spontaneous behavior, can also be influenced by other factors not directly related to pain such as motivation (Sherwin et al., 2004), fear, anxiety, distress, suffering, olfactory irritants and aversive situations, or generally by depressed general condition and changes in overall wellbeing. Therefore, control experiments were conducted to distinguish between the impact of anesthesia and analgesia *per se* and of surgery as the pain-inducing insult. To estimate the impact of pain, surgery was conducted either with or without pain relief. Moreover, care was taken to prevent any other surrounding factors unrelated to pain from influencing the experiments, i.e., the animal room was insulated and animals were not disturbed by the presence of any personnel in the post-operative time frame in which video recording was performed and burrowing took place.

The burrowing setting described previously by Deacon was adapted in our approach as we used smaller tubes and carried out the test at a different time of day. These modifications had negligible influence, since we had found in a preliminary experiment in our laboratory (data not shown) that burrowing performance, especially the latency to burrow, was largely independent of circadian rhythm, i.e., the time of day of testing. Animals were allowed to habituate to the test apparatus during the acclimatization period since Deacon showed that healthy mice increased burrowing activity at the second trial and then continued their high level of burrowing (Deacon et al., 2001). In another, not yet published, study using this test repeatedly, we found only slight oscillations of baseline burrowing performance, but no tendency toward longer or shorter latencies to burrow or burrowing duration (data not shown). To exclude disturbances that will induce fear, agitation, or any kind of distress, and thus certainly have impact on the animals' spontaneous behavior, we avoided any human presence in the critical time frame, and no-one handled cages or animals during this time. Hence, for the appraisal, the pellets were not weighed at a predetermined time point (i.e., after 2 h; Deacon et al., 2001), but rather the weight of the removed pellets was calculated by counting them from video recordings at a certain time point. This procedure gave results comparable to those known from

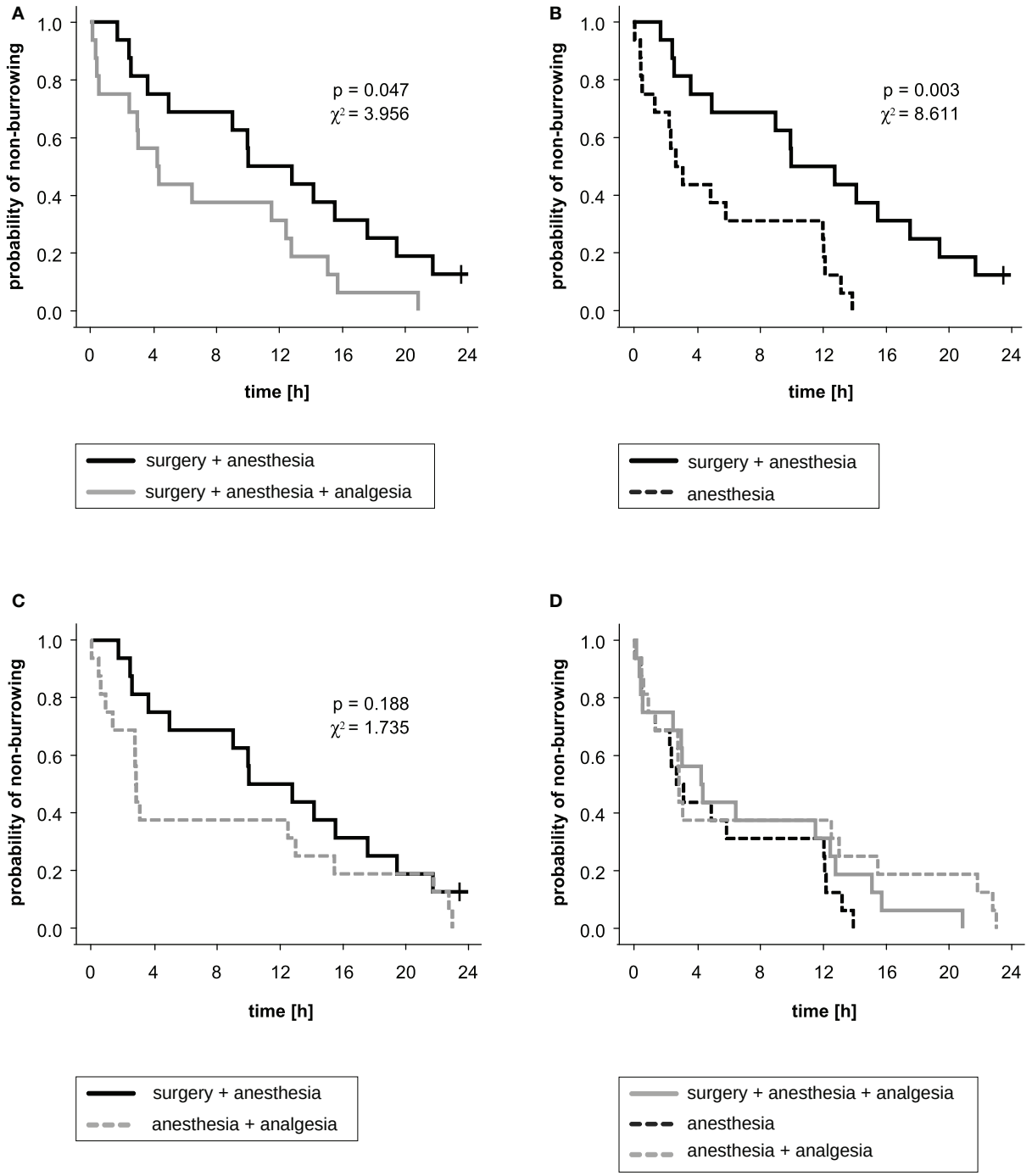

FIGURE 4 | Kaplan–Meier analysis and results of log rank significance test of latency to burrow. The curves indicate the probability that a mouse with a given treatment has not yet started to burrow. The probability of non-burrowing (*y*-axis) is traced against the time to event in hours (*x*-axis). **(A)** Significant difference between groups treated (or not) with an analgesic for surgery. **(B)** Significant difference between groups of mice that underwent anesthesia and surgery without pain relief and mice that were anesthetized only. **(C)** The difference between the surgery + anesthesia group and the anesthesia + analgesia group was not statistically significant. **(D)** No significant differences in latencies to burrow were found between surgery + anesthesia + analgesia, anesthesia and anesthesia + analgesia groups.

the literature (Deacon et al., 2001). Thus, with the advantage of video recording, we could follow the activities of each mouse in its home cage without disturbing the animal by handling the tube. The infrared-sensitive video recording allowed us to determine the latency to burrow accurately, and to define the time frame over which differences in burrowing performance were greatest between

groups receiving different treatments. In this way we could show that less than 30% of mice that had undergone surgery without pain alleviation had started burrowing 7–9 h post-operatively. In contrast, most animals in which a pain killer was administered during surgery (62%), as well as those mice that received only anesthesia (70%) or anesthesia and analgesia (62%), had started burrowing by 7–9 h after surgery.

As the construction of burrows offers advantages as protection from natural threats (e.g., predation, detrimental environmental conditions) in the wild, burrowing is highly motivated behavior, and persists as spontaneous behavior during domestication in the laboratory mouse (Adams and Boice, 1981; Dudek et al., 1983; Sherwin et al., 2004). As burrowing is not essential for mice in the laboratory environment, the motivation to perform the behavior may result from its reinforcing consequences. Decreased engagement in activities like burrowing may be seen as an alteration in motivation to engage in non-essential but self-rewarding activities (Cunningham et al., 2007; Teeling et al., 2007), which can be induced by various impacts on the animals' general condition and wellbeing, such as disease, pain, distress, or other impairment. Thus, the results of burrowing performance testing imply that there might be a balance between the motivation to burrow and an individuals' depression of general condition or level of pain experienced. During the animals' recovery from the noxious insult and the regeneration of wellbeing, the motivation to burrow will prevail over pain and impairment. Thus, the latency to burrow could act as an indicator of the duration of pain and reduced wellbeing in the individual. This interpretation of burrowing performance is supported by other studies hinting at an approximate time frame regarding the possible duration of pain in the laparotomy model (Arras et al., 2007; Langford et al., 2010).

In general, the prolongation of latency to burrow was in clear accordance with previous results from telemetric measurements of heart rate and heart rate variability values, as well as with decreased body weight progression and food consumption in an almost identical laparotomy model conducted in our laboratory (Arras et al., 2007). From our former study and from the findings presented here, post-operative pain is suggested as being the cause of the alterations in physiology and behavior, although symptoms of pain were not apparent upon short-term (2–10 min) observation of the appearance, posture, and spontaneous locomotion of the animals. Such absence of obvious signs of pain after minor and moderate surgery in laboratory mice is commonly known from anecdotal evidence and from the experiences in the worldwide laboratory routine of embryo transfer and vasectomy, which both normally include laparotomy. Since treatment with a pain killer restored the burrowing performance to very close to the level of the control groups (anesthesia only, anesthesia + analgesia), pain seems the most likely reason for the decreased burrowing behavior.

As other studies found a decrease in burrowing activity without a corresponding decrease in Open Field activity (Teeling et al., 2007), it seems unlikely that a pure mechanical impairment caused the observed prolongation of latency to burrow after surgery. While this study cannot deliver proof of pain in the laparotomy model, and the impact of anesthesia (as seen in the anesthesia only group) should not be underestimated, our results, together with cited literature observations, again support pain as the explanation for decreased burrowing behavior, while simultaneously suggesting burrowing behavior as a sensitive and easy to observe indicator of pain in laboratory mice. Since burrowing is an operant behavior, it may also be useful for testing of supraspinal drug targets, in contrast to the mainly peripheral acting analgesic we used here.

One limitation of our study is that we have analyzed systematic data only in the most commonly used C57BL/J inbred strain. However, burrowing testing has been shown to be effective in several inbred strains of laboratory mice (Solberg et al., 2006, our own preliminary data), and also works in principle also in rats, hamsters, and gerbils (Deacon, 2009), which leads us to suggest that our setting would work equally well in other mouse strains. In addition, our setup focused on the assessment of the post-operative condition of mice after laparotomy. The reproducibility of the experimental setting and the critical measuring window of 7–9 h might be proved in the future in other models of surgery or other interventions that can induce persistent pain, noxious stimuli of moderate duration, or impairment of general condition and wellbeing.

In summary, the impact of laparotomy on the general condition and wellbeing of laboratory animals was assessed using a test setting based on the natural burrowing behavior of mice. The setting was easy and convenient to perform in the animal's home cage under routine laboratory conditions. As the results of our tests allowed us to discriminate between groups of mice that were treated with a pain killer for surgery or not, burrowing performance appears to be correlated with post-operative pain, and may give hints on its duration in individual mice.

This practical setting could be tested in the future in other surgical models and in other strains of mice as a standard evaluation method for post-operative depression of general condition and wellbeing, including mild-to-moderate degrees of post-operative pain. The reproducibility of the method in other approaches and its value as a test in basic research into the mechanisms and pathophysiology of pain, and in the searching for novel pain killers is a promising avenue of research for further study.

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Chapter 3: Burrowing is a sensitive behavioural assay for monitoring general well-being during DSS colitis in laboratory mice

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Burrowing is a sensitive behavioural assay for monitoring general wellbeing during dextran sulfate sodium colitis in laboratory mice

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Abstract

An impaired intestinal epithelial barrier is thought to be a major factor in the pathogenesis of human inflammatory bowel disease (IBD). IBD is frequently investigated by inducing a damaged barrier in murine models of colitis. This can be done by feeding mice with dextran sulfate sodium (DSS) polymers in their drinking water. Refinement measures should focus on alleviating unnecessary suffering during this probably painful condition. Appropriate parameters are needed to decide when to terminate the experiments. Our aim was to investigate whether a change in burrowing behaviour is a sensitive measure of animal welfare in murine models of colitis. Acute colitis was induced in C57BL/6 mice with 2.0% DSS over nine days. The burrowing test is based on the species-typical behaviour of mice to spontaneously displace items from tubes within their home cage. As a burrowing apparatus, a water bottle (250 mL, 150 mm length, 55 mm diameter) filled with 138–142 g of pellets of the animal's diet was used. The presence of intestinal inflammation as a result of acute DSS-induced colitis was confirmed by a decrease in body weight, colon length and an increase of murine endoscopic index of colitis severity, histological score and spleen weight in the group receiving DSS as compared with the control group. An onset of intestinal inflammation correlated with a significant decrease in burrowing behaviour ($P < 0.05$). Altered adrenal gland histology indicated stress as a result of acute colitis. Our findings provide evidence that changes of spontaneous burrowing behaviour correlate with the onset of inflammation in acute DSS-induced colitis.

Keywords

dextran sulfate sodium (DSS), colitis, burrowing, pain, behaviour, mice, inflammation, animal wellbeing

Laboratory mice are currently a widely-used animal species in biomedical research. Their current popularity may be due to the availability of a multitude of spontaneous or experimentally-induced mutants, allowing studies of in vivo functions of single genes. In recent years an increase in the use of genetically-modified mice in research has been observed.[1] Mice are also used to test hypotheses concerning the aetiology and pathogenesis of inflammatory bowel disease (IBD). IBD is a chronic, relapsing inflammation of unknown origin. IBD comprises two main disease conditions, ulcerative colitis (UC) and Crohn's disease (CD). Disturbance of the epithelial barrier and epithelial transport processes has been proposed as a major factor in the pathogenesis

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of IBD. Epithelial apoptosis in mucosa from patients with UC[2] and CD[3] was found to be considerably upregulated followed by increased permeability of the epithelial barrier.[4–6] Abnormal gut permeability is associated with the translocation of luminal antigens, intestinal bacteria and endotoxins.

The consequences of a disturbed barrier in human IBD are frequently investigated in mouse models of impaired epithelial cell function. Feeding mice for several days with dextran sulfate sodium (DSS) polymers in the drinking water induces acute colitis characterized by bloody diarrhoea, ulcerations and infiltrations of the intestinal mucosa with granulocytes.[7] It is generally believed that DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier.[8] DSS-induced colitis is readily reproducible in the common C57BL/6 strain, which shows intermediate to high susceptibility to DSS colitis but low mortality rates.

Appropriate parameters for monitoring the induction of colitis and impact on the wellbeing of mice are needed to decide when to terminate the experiments. On the one hand, a certain level of inflammation is necessary for observing the colitis-specific disease pattern, but on the other hand, animal wellbeing needs to be considered during the probably painful condition. While a certain level of inflammation is necessary for observing the colitis-specific disease pattern; for ethical reasons, distress from this probably painful condition should be minimized. Humane endpoints must be set to avoid unnecessary suffering in these mice. Therefore, appropriate parameters for monitoring the induction of colitis and the impact of intestinal inflammation on the wellbeing of mice are needed to decide when to terminate the experiments.[9,10]

To assess the grade of induced colitis in a DSS-treated animal, a retrospective histological examination of affected colon parts is usually performed, which requires sacrificing the animals. In the live animal, monitoring of the course of colitis and the progressing inflammation of the colon can be achieved via endoscopic examination in the end stage of the disease process or by observation of body weight loss. While endoscopy is associated with anaesthetic immobilization of the animal and may therefore be linked with physiological and emotional stress, the assessment of body weight loss alone may be a problematic parameter because of its retrospective character.[11] Changes in body weight can be associated with several impairments of the animal's condition and therefore represent a relatively unspecific indicator, especially as animals suffer from diarrhoea during progressing colitis. For indication of impaired wellbeing or pain, body weight loss is therefore only relevant to a limited extent.[11] This situation requires additional parameters for the assessment of induced colitis, as well as animal wellbeing. Detection of pain and reduction of wellbeing in DSS-treated animals is essential for both scientific and ethical reasons. However, pain diagnosis in mice is challenging as mice often exhibit only subtle signs of pain, suffering or disability.[11–14]

A promising, simple approach to address this need is the assessment of changes in spontaneous burrowing behaviour.[15] The test is based on the species-typical behaviour of mice to spontaneously displace items from tubes within their home cage. Animals show some tendency to increase their burrowing with experience but these changes are not statistically significant.[16] Burrowing behaviour has been used as a tool to provoke and determine burrowing behaviour under experimental conditions in models of prion disease (scrapie),[17–20] brain lesions[21] and complex regional pain syndrome[22] in C57BL/6 mice, lipopolysaccharide (LPS)-induced low-grade systemic inflammation[21] using C3H/HeJ and C3H/NeJ mice, and Alzheimer's disease using BL6/SJL mice.[23] In these models, a decrease in burrowing behaviour was interpreted as being correlated with chronic neurological or immunological disorders and was very efficient in detecting early signs of the diseases mentioned. Recently changes in burrowing behaviour were found to be sensitive indicators of post-operative pain after laparotomy in mice.[24] As burrowing behaviour is not an essential maintenance behaviour like food consumption or sleeping,[25] we suggest that it is sensitive to different, subtle kinds of impact on the animals' general condition and wellbeing. Burrowing is a species-typical behaviour of mice.[26] Burrowing as a screening test for therapies may therefore offer great sensitivity, along with cheapness and simplicity. Changes in this species-typical behaviour might therefore be useful as an indicator of stress and pain under pathological conditions like induced colitis. We therefore investigated changes in species-typical burrowing behaviour during experimental colitis.

Materials and methods

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under licence no. 149/2009, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe no. 123, Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Table 1. Murine endoscopic index of colitis severity (MEICS).

	0	1	2	3	Total
Thickening of the colon	Transparent	Moderate	Marked	Non-transparent	0–3
Changes of the vascular pattern	Normal	Moderate	Marked	Bleeding	0–3
Fibrin visible	None	Little	Marked	Extreme	0–3
Granularity of the mucosal surface	None	Moderate	Marked	Extreme	0–3
Stool consistency	Normal + solid	Still shaped	Unshaped	Spread	0–3
					Overall: 0–15

Endoscopic colitis score based on the observed signs of inflammation. The MEICS consisted of five parameters, as indicated.

Animals

Twenty-three C57BL/6 mice weighing 20–22 g were obtained from our in-house breeding facility (Fuellinsdorf, Switzerland) at the age of nine weeks. Female mice were used as male mice frequently show aggressive behaviour upon DSS treatment. All the animals were housed for at least three weeks prior to testing in a specific pathogen free (SPF) facility. The animals were randomly assigned to cages and kept in groups of two (with the exception of one group of three) in type II long clear-transparent individually ventilated cages (IVCs, 365mm × 207mm × 140mm, Allentown, New Jersey, USA) with autoclaved dust-free bedding and tissue papers as nesting material. They were fed a pelleted and extruded mouse diet (R/M–H Extrudat, ssniff Spezialdiäten, Soest, Germany) ad libitum and had unrestricted access to sterilized drinking water. The light/dark cycle in the room was given through natural daylight (sunrise: 07:00 h, sunset: 18:00 h). The mice were weighed at 10:00 h every morning; burrowing tests were started 2 h prior to sunset. The temperature was set to $21 \pm 1^{\circ}$C, with a relative humidity of $55 \pm 5\%$ and 75 complete changes of filtered air per hour (filter: Megalam MD H14, Camfil, Zug, Switzerland).

Induction and treatment of DSS colitis

Mice were randomly assigned to the cages and acute colitis was induced in 12 female C57BL/6 mice by feeding 2% DSS in drinking water over nine days as described previously.[7,8] The colonic inflammation resulting from DSS depends on a number of unknown factors, e.g. the microbiome present. For the mice in our SPF facility we chose to induce acute colitis by feeding 2% DSS in drinking water over nine days. Reduced food consumption and an unshaped or spread stool consistency can frequently be observed upon DSS induction. Both contributed to body weight loss $< 20\%$ for the conditions present in our SPF facility. Body weight was determined by weighing the animals using a precision balance (PR 2003 Delta Range, Mettler-Toledo AG, Greifensee, Switzerland) especially designed for weighing moving animals. Stool consistency is part of the colonoscopy score described below. Eleven mice not fed with DSS were used as controls. All the animals were sacrificed on day 9 by cervical dislocation.

Assessment of colonoscopy score in mice

Animals were anaesthetized intraperitoneally with a mixture of 90–120 mg ketamine (Narketan 10%, Vétoquinol AG, Bern, Switzerland) and 8 mg xylazine (Rompun 2%, Bayer, Lyssach, Switzerland) per kg body weight and were examined as described previously.[27] The solid endoscope was introduced per anus with a lubricant (2% lidocaine, Kantonsapotheke Zurich, Switzerland). The colon was cautiously inflated with air. As the colonoscope was gently advanced into the colon, the abdomen was observed to localize the tip of the endoscope by transillumination. The endoscope could easily be passed at least 4 cm into the colon. Recording was performed using the Karl Storz Tele Pack Pal 20043020 (Karl Storz Endoskope, Tuttlingen, Germany). Colonoscopy was scored on day 9 by the murine endoscopic index of colitis severity (MEICS) as previously described.[27] The scoring system is shown in Table 1.

Assessment of histological score in mice

The first distal centimetre of the colon was removed and used for histological analysis. The mice were scored individually by an independent investigator blinded to the type of treatment. The total histological score represented the sum of the epithelial and infiltration score, and thus ranged from 0 to 8 (maximum total score). Histology was scored as follows:

- Epithelium: 0 = normal morphology; 1 = loss of goblet cells; 2 = loss of goblet cells in large areas; 3 = loss of crypts; 4 = loss of crypts in large areas.
- Infiltration: 0 = no infiltrate; 1 = infiltrate around crypt basis; 2 = infiltrate reaching to the L. muscularis mucosae; 3 = extensive infiltration reaching the

L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4 = infiltration of the L. submucosa.

Behavioural testing with burrowing apparatus

As burrowing apparatus, a plastic bottle (standard opaque water bottle, 250 mL, 150 mm length, 55 mm diameter) filled with 138–142 g of pellets of the animal's diet was used. An empty bottle of the same dimensions was provided to serve as a shelter for the animals.[24] To test for effects of DSS-induced colitis on burrowing behaviour each animal was tested in the burrowing apparatus before (baseline) and during the DSS administration (experiment). The individual baseline values were used to compensate for inter-individual variation in burrowing behaviour. For acclimatization, the animals were housed in groups of two mice for three days in a standard IVC containing the experimental set-up as described above. Except for this prior experience with the burrowing apparatus, the animals had no experience with behavioural testing and were not selected for burrowing performance.

Burrowing tests for the measurements of baseline and experimental values of burrowing behaviour started 2 h before sunset and were carried out in the animal room. Pellets which had been removed or eaten by the mice during the preceding days were replaced in the apparatus before testing started. The burrowing apparatus was weighed 2 h after the start of the experiment to assess the weight of the removed pellets. The apparatus was again placed in the cage after the removed pellets were replaced and weighed 12 h later at the end of the dark phase. On day 9, the burrowed weight was additionally evaluated after 0.5 h. Burrowing tests were performed on days 4–9 of the DSS administration.

Hyperplasia and hypertrophy in adrenal gland

To assess cellular hyperplasia and hypertrophy in the adrenal cortex and medulla, paraffin-embedded sections were haematoxylin and eosin (H&E) stained as described above. Cell nuclei in the zona glomerulosa (ZG) and zona fasciculata (ZF) were counted in defined areas (5 regions each from 20 different mice) at 20-fold magnification. Numerical density (labelled cells per volume) was determined. An increase in nuclear density in the adrenal cortex was interpreted as evidence of a decreased cell size, whereas a decrease in nuclear density was interpreted as evidence of an increased cell size.[28,29] In all cases, care was taken to avoid sampling regions of the medulla that contained large blood vessels, because this would have greatly affected cellular density measurements.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney rank sum test. In weight curves, mean values ± SEM are displayed. Box plots express median, 25% quartiles, minimum and maximum. Differences were considered significant at a P value of <0.05 (*), highly significant at a P value of <0.01 (**) and very highly significant at a P value of <0.001 (***).

Results

DSS colitis negatively alters the colonic mucosa at a macroscopic level

To determine changes in burrowing behaviour during the onset of intestinal inflammation we used the DSS colitis mouse model. During induction of acute colitis, the mice received 2% DSS dissolved in drinking water ($n = 12$) or drinking water alone ($n = 11$) over nine days. Water consumption was not reduced in mice treated with DSS. Weight loss in acute colitis was determined over nine days (Figure 1). The percentage changes of body weight in the group receiving DSS were significantly different from the control group on day 7 ($98.6 \pm 3.1\%$ versus $101.8 \pm 2.5\%$, $P < 0.05$), day 8 ($94.9 \pm 7.2\%$ versus $103.1 \pm 3.8\%$, $P < 0.01$) and day 9 ($91.4 \pm 5.7\%$ versus $104.6 \pm 2.9\%$, $P < 0.001$).

Macroscopic mucosal damage was assessed by endoscopy and colonoscopy score. During colonoscopy

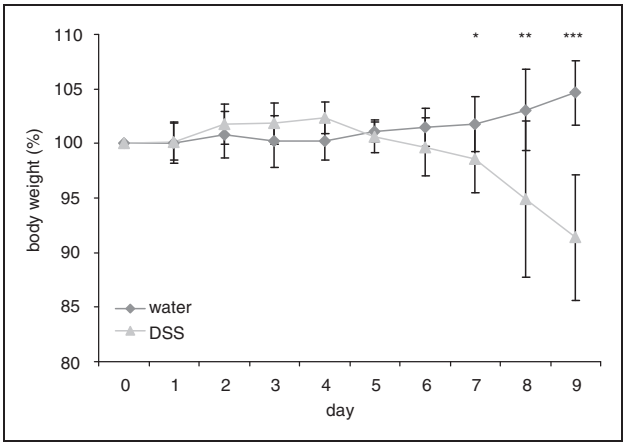

Figure 1. Percentage body weight loss. Mice received either dextran sulfate sodium (DSS) (triangles, $n = 12$) or water (diamonds, $n = 11$). Induction of colitis was followed by a significant reduction of the weight. Bars represent mean ± SD. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

on day 9 mucosa from control mice displayed a smooth and transparent mucosa with a normal vascular pattern (MEICS 2.0 ± 0.4, $n = 11$, Figure 2a and b). A solid stool was visible. By contrast, mucosa from animals with DSS colitis appeared with an intransparent mucosa and altered vascular pattern (MEICS 12.0 ± 0.5, $n = 12$, $P < 0.01$; Figure 2c and d). The mucosal surface appeared more granular than with mice without DSS colitis, and the thickening of the colon was more prominent. Diseased regions often had a cobblestone-like appearance and casually mucosal bleeding. Unshaped stool was visible. The endoscopic colitis score indicated severe mucosal damage in the DSS colitis mice.

Induction of colitis was also followed by a significant reduction of the colon length as compared with the control animals (6.4 ± 0.6 cm versus 9.4 ± 0.5 cm, $P < 0.001$; Figure 3). Additionally, the spleen weight, normalized to the body weight, was significantly increased during acute colitis as compared with the control animals (5.4 ± 0.6 mg/g versus 4.7 ± 0.4 mg/g, respectively, $P < 0.001$; Figure 4). Both these parameters

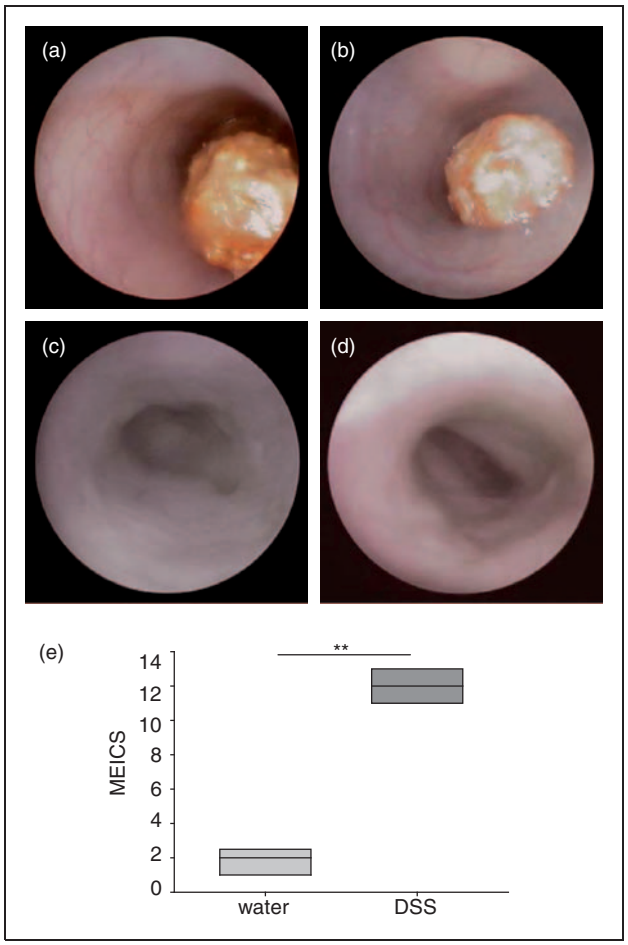

Figure 2. Endoscopic images of dextran sulfate sodium (DSS) colitis and murine endoscopic index of colitis severity (MEICS) (day 9). Control mice (a and b). Mice upon DSS treatment (c and d). Control mice showed no signs of inflammation and solid stool compared with a thickened colon and granular mucosal surface upon DSS induction. Images are representative of five mice. MEICS (e). Statistical analysis was performed using the Mann–Whitney rank sum test. Box plots express median, 25% quartiles, minimum and maximum. $^{**}P < 0.01$.

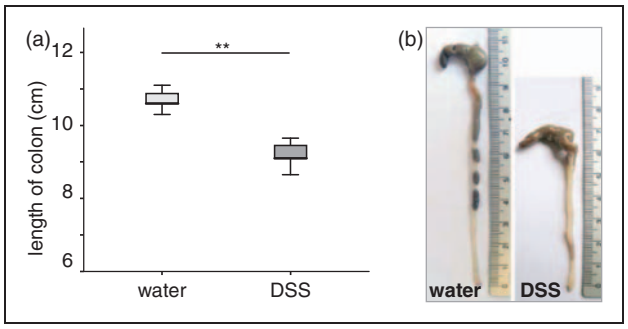

Figure 3. Colon lengths. (a) Mice received either dextran sulfate sodium (DSS) ($n = 12$) or water ($n = 11$). Induction of colitis was followed by a significant reduction of the colon length. Statistical analysis was performed using the Mann–Whitney rank sum test. Box plots express median, 25% quartiles, minimum and maximum. $^{**}P < 0.001$. (b) Colon of a mouse which received water (left) or DSS (right). Images are representative of 12 and 11 mice, respectively.

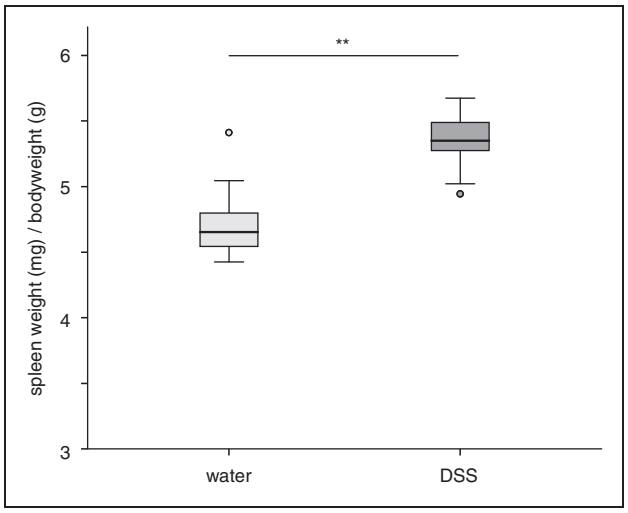

Figure 4. Spleen weights. Mice received either dextran sulfate sodium (DSS) ($n = 12$) or water ($n = 11$). Induction of colitis was followed by a significant increase of the spleen weight. Statistical analysis was performed using the Mann–Whitney rank sum test. Box plots express median, 25% quartiles, minimum and maximum. $^{**}P < 0.01$.

demonstrated the induction of a severe inflammation in response to DSS treatment.

DSS colitis negatively alters the colonic mucosa at a microscopic level

The histological score for the mice receiving DSS was significantly increased compared with that of the water-treated animals (7.5 ± 0.5 versus 0.4 ± 0.4, respectively, $P < 0.001$; Figure 5a). Colonic sections taken from the mice with DSS colitis showed a loss of crypts, extensive epithelial damage, and both infiltration and thickening of the mucosa over large areas (Figure 5d and e) compared with that of the water controls (Figure 5b and c). These data show that in mice with DSS colitis the colonic mucosa is also negatively altered at a microscopic level.

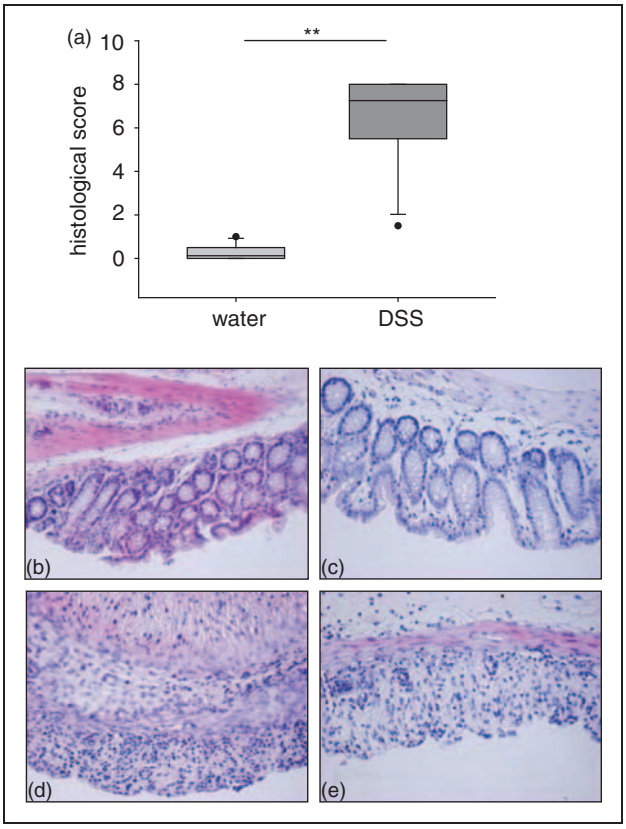

Figure 5. Histological parameters and H&E staining of terminal colon. (a) Mice received either dextran sulfate sodium (DSS) ($n = 12$) or water ($n = 11$). Induction of colitis was followed by a significant increase of the histological score. Statistical analysis was performed using the Mann–Whitney rank sum test. Box plots express median, 25% quartiles, minimum and maximum. $^{**}P < 0.005$. (b–e) H&E staining. Control mice (b and c) were treated without DSS. Taken from mice with DSS-induced colitis (d and e). Control mice showed no signs of inflammation and an intact epithelial barrier compared with a thickened mucosa and loss of crypts upon DSS. Images are representative of 12 mice.

Onset of intestinal inflammation correlates with decrease in burrowing behaviour

The burrowing test (modified from descriptions by Deacon et al.[30]), was evaluated in regard to its feasibility as a means to assess general condition and presumable pain in animals suffering from experimentally-induced colitis. The test proved to be easy to perform in an experimental setting within the animals' home cage and burrowing performance responded sensitively to the DSS treatment. We observed that healthy mice started burrowing (i.e. displaced substantial numbers of food pellets from a tube) in general within several minutes after this pellet-filled tube was provided to them, while mice treated with DSS started burrowing later or not at all. The measured weight of removed pellet material in the default time frame of 2 and 12 h (referred to by Deacon et al.[30]) was significantly reduced during the treatment ($P < 0.05$ each).

Figure 6 presents these changes in percentage weight of removed pellets by two mice after 2 h and 12 h of testing during the experimental period. During baseline measurements after 2 h and 12 h each pair of mice burrowed a mean weight of 70.7 ± 10.8 g and 83.5 ± 2.7 g, respectively. While the control animals showed only a slight decrease in burrowing performance during the following days of observation, the DSS-treated mice removed fewer pellets during the progression of colitis. Compared with the control animals, the mice treated with DSS removed significantly fewer pellets after 2 h on day 8 (49.2 ± 28.8 g versus 79.6 ± 6.8 g per two mice, $P < 0.05$) and day 9 (37.7 ± 35.1 g versus 75.4 ± 8.7 g per two mice, $P < 0.05$, Figure 6a). After 12 h, the weight of pellets removed by the DSS-treated mice was significantly lower than that of the control group on day 8 (64.2 ± 11.9 g versus 80.2 ± 2.2 g per two mice, $P < 0.01$, Figure 6b). On day 9 a very similar trend was observed. Additional measurements after 0.5 h on days 8 and 9 revealed a significant decrease in pellets removed by the DSS-treated animals as compared with the water controls (13.7 ± 24.7 g versus 65.8 ± 26.4 g per two mice, $P < 0.05$, and 16.5 ± 25.6 versus 62.2 ± 20.2 g per two mice, respectively, $P < 0.05$).

An altered adrenal gland suggests onset of stress upon DSS-induced colitis

Chronic stress induces changes in adrenal growth and function. Therefore we determined whether treatment with DSS is associated with cellular hypertrophy in the medulla and/or decreased cell size in the cortex of

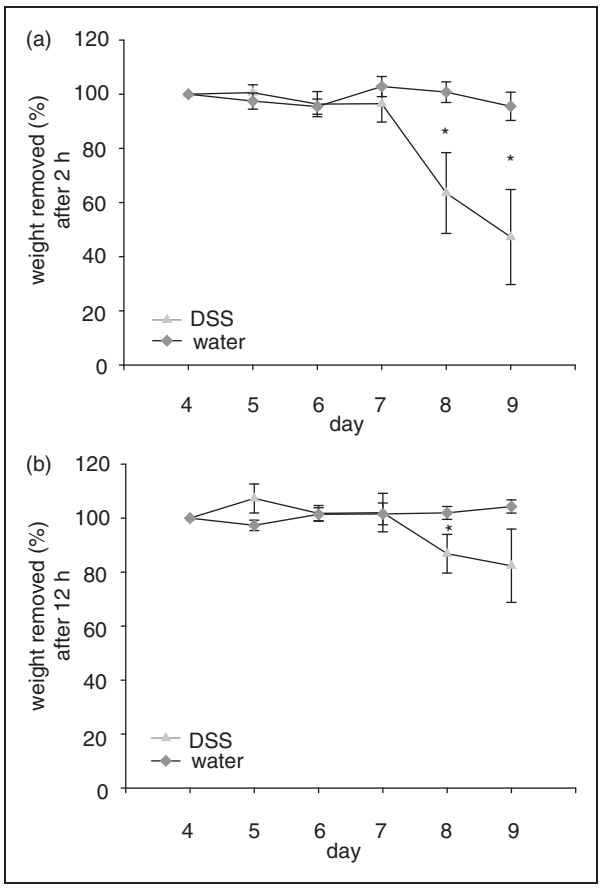

Figure 6. Burrowing test. Percentage weight of pellets removed by two mice out of test apparatus. Mice received dextran sulfate sodium (DSS) (triangles, $n=6$) or water (diamonds, $n=5$). Burrowing was tested for 2 h (a) and 12 h (b). Induction of colitis was followed by a significant reduction of the weight of the removed pellets. Mann–Whitney rank sum test was performed. Bars represent mean ± SEM, $*P<0.05$.

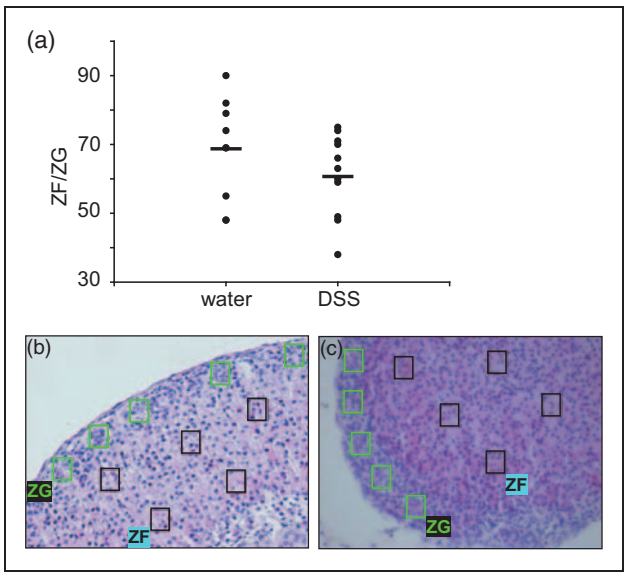

Figure 7. Hyperplasia and hypertrophy in adrenal gland. (a) Adrenal sections collected from mice which received either water ($n=11$) or dextran sulfate sodium (DSS) ($n=9$). Induction of colitis was followed by a decrease of the zona fasciculata (ZF)/zona glomerulosa (ZG) ratio. (b) H&E staining of an adrenal gland section from a mouse which received water (representative of 11 mice). (c) DSS colitis induced cellular hypertrophy in the inner ZF and medulla, compared with a decreased cell size in the ZG (representative of nine mice). Nuclei were counted in five areas in both the ZG (green boxes – see online version for colour reference) and the ZF (black boxes) and summed up for result.

adrenal glands. Induction of colitis was followed by a decrease of the ZF/ZG ratio (mean ± SD: 68 ± 15 versus 61 ± 12, Figure 7a). Nuclear density in the ZG was increased in the control mice (Figure 7b) compared with the mice that received DSS (Figure 7c). In the adrenal medulla DSS decreased nuclear density was found, suggesting increased cell size in this region.

Discussion

In this study we have suggested a simple but sensitive behavioural test system for assessing the impact of colitis on animal wellbeing. We used acute DSS-induced colitis to induce intestinal inflammation. Parameters collected during the induction of acute DSS-induced colitis documented the onset of inflammation in mice. A significant decrease in body weight and a significant increase of MEICS in the group receiving DSS compared with the control group indicated mucosal damage in the DSS colitis mice. Parameters collected retrospectively confirmed the presence of inflammation. A significant decrease in colon length as well as a significant increase in both histological score and spleen weight for animals receiving DSS compared with the control group indicated the induction of inflammation in response to DSS treatment. As expected, DSS colitis also negatively altered the colonic mucosa at both macroscopic and microscopic levels. The murine model of acute DSS-induced colitis is comparable with acute colitis in human patients. Animals completely recover from acute colitis after discontinuing DSS without chronic manifestation of inflammation. This includes weight gain, decrease of MEICS, normalization of colon length and decrease of histological score and spleen weight. Acute DSS-induced colitis does not affect the survival rate.

In parallel we investigated the changes in burrowing behaviour under these conditions. The onset of intestinal inflammation indicated by a weight loss in DSS-treated mice compared with the controls on days 7, 8 and 9 correlated with a reduction of burrowing

performance on days 8 and 9. DSS-treated mice removed significantly fewer pellets during the progression of colitis compared with their respective control animals. Behavioural testing with the burrowing apparatus provided reliable results when an experimental period of 0.5 or 2 h was applied. In our experimental set-up two mice were kept in a cage during the burrowing test. Acute DSS-induced colitis, just as any other induced disease, does not develop in each mouse with the same onset or severity of inflammation. To identify outliers animals need to be housed separately for the duration of the burrowing test. Further, we determined whether treatment with DSS is associated with cellular hypertrophy and decreased cell size in the adrenal glands. An altered adrenal gland is an indication of first signs of chronic stress upon acute DSS-induced colitis at a cellular level. This suggests that altered burrowing behaviour could also be a sensitive readout parameter for nascent chronic stress.

A correlation between inflammation and burrowing as well as pain and burrowing has been shown in a number of recent studies. The immune system communicates with the brain in times of both good health and illness. Inflammation can be associated with behavioural changes, collectively referred to as sickness behaviour, characterized by symptoms such as cognitive dysfunction, anxiety, depression and lethargy. Injection of double-stranded RNA into C57BL/6 mice mimics an acute phase of viral infection. This treatment induced severe sickness behaviour in animals as revealed by a burrowing test performed 6 h post injection.[31] Low-grade systemic inflammation was induced in mice using LPS to mimic aspects of bacterial infection. Sub-pyrogenic inflammation resulted in changes in burrowing.[21] Further, in a mouse model of chronic neurodegeneration additional transient systemic inflammation by injection of LPS led to an increase of inflammatory markers like IL-1β, TNF and IFN-β. This inflammation caused exaggerated impairments in burrowing and locomotor activity.[32] Selective and non-selective inhibitors of cyclooxygenase (COX)-1 like piroxicam, indomethacin and ibuprofen reversed the effect of LPS-induced systemic inflammation on behaviour. This suggests that inflammation-driven changes in burrowing depend on COX-1.[33]

As the construction of burrows provides protection from natural threats (e.g. predation and detrimental environmental conditions) in the wild, burrowing is a highly motivated behaviour persisting as a spontaneous behaviour in the laboratory mouse.[25,34,35]

Knowledge of disease progression during acute DSS-induced colitis is of immediate importance for establishing humane endpoints and for monitoring wellbeing. In studies based on this model useful indicators of the onset of inflammation, disease progress and pain are rare. Directly accessible parameters like appearance, common behaviour, overall activity, stool consistency, visible blood on stool and prolapse formation must be determined upon DSS-induced colitis to comply with basic ethical needs. But a mouse without overt pathological findings is neither necessarily free of pain nor reliably non-inflamed. Colonoscopy should not be performed on a daily basis because of the risk of mucosal perforation. Cytokine levels could be determined from peripheral blood but blood should also not be taken daily. The inflammatory parameter calprotectin present in neutrophils in mice can be determined from stool samples. But as inflammation is mainly initiated by epithelial lesions in the mentioned model, calprotectin is often not a reliable parameter in acute DSS-induced colitis. Therefore body weight loss is the only physical indicator for the onset of inflammation that can be measured repeatedly. However, body weight may also be dependent on specific treatments, e.g. during nutrition or pharmacological trials. Further this could play a major role if long-term models of colitis were used. Chronic manifestation of inflammation can be initiated using the murine model of chronic DSS-induced colitis. Here the administration of DSS for several cycles (e.g. 7 days DSS, 14 days water) results in chronic colitis after a time period of two to three months. Despite body weight being a robust physical parameter it is of only limited use for determining impaired wellbeing or pain.[11] Behaviour, like burrowing performance, in contrast to most physiological and clinical parameters, can be observed easily in a non-invasive manner and has been described as a promising approach to assess animal wellbeing.[15,36–39] Here we provide evidence that testing burrowing performance could be a sensitive behavioural test system for assessing the impact of colitis on animal wellbeing. Our data indicate that, next to body weight loss and MEICS, burrowing behaviour might be a useful parameter that can be easily documented during the induction of DSS colitis to confirm the onset of inflammation in mice.

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Declaration of conflicting interests

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Chapter 4: Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring

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Assessment of postsurgical distress and pain in laboratory mice by nest complexity scoring

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Abstract

Preliminary studies have suggested a correlation between postsurgical pain and nest building behaviour in laboratory mice. However, there is no standardized measure for estimating pain by means of nest building performance. Here, we investigated nest building under various conditions, and scored nest complexity to assess postsurgical pain. Mice of both sexes, different strains [C57BL/6J, DBA/2J, and B6D2-Tg(Pr-mSMalphaActin)V5rCLR-25], and kept under different housing conditions, showed no differences in their latency to use the offered nest material. Healthy female C57BL/6J mice were engaged 4.3% of the day with nest building and showed three peaks of this behaviour: in the beginning and middle of the light phase, and in the second half of the dark phase. For assessment of postsurgical pain, female C57BL/6J mice underwent a sham embryo transfer +/− different doses of the analgesic carprofen or control treatment. Nest complexity scoring at 9 h after the experimental treatments (i.e. at the end of the light phase) resulted in less than 10% of animals with noticeably manipulated nest material (nestlet) after surgery and more than 75% of healthy mice having built identifiable-to-complex nests or had noticeably manipulated nestlets, while animals after anaesthesia-only showed intermediate nest complexity. Carprofen analgesia resulted in no (5 mg/kg) or only slight (50 mg/kg) improvement of nest complexity after surgery. Thus, nest complexity scoring can be incorporated into daily laboratory routine and can be used in mice as a sensitive tool for detecting reduced wellbeing and general condition, but probably not for determining the efficacy of pain treatment.

Keywords

Mice, nest building, pain assessment, nest complexity scoring, refinement

The construction of nests is common in rodent species. Wild house mice build nests to provide heat conservation; shelter from elements, predators, and competitors; and to allow successful reproduction.[1–3]

The motivation and ability to perform this complex behavioural sequence culminating in a finished nest persist also in domesticated mice and those in laboratory animal facilities. Aside from 'brood' or maternal nests, built specifically for reproduction, if provided with suitable nest building materials, laboratory mice of both sexes build 'sleeping' or non-maternal nests.[4,5] In the laboratory setting, nests might allow the mouse to shield itself from conspecifics, as well as humans and external stimuli, e.g. direct light.[6] Also, as most animal facilities have ambient temperatures below their thermoneutral temperature, mice might build nests for thermoregulatory reasons.[7,8] The motivation for nest building is high, and nest building material is highly valued by laboratory mice.[9,10]

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Spontaneous, species-specific behaviours, such as nest building, that are performed in the animals' home cage have been proposed in the last decade as useful indicators for welfare assessment in small laboratory animal species, and might be used as simple, non-invasive and reproducible indicators for estimating, e.g. neurological dysfunction or pain. A reduction in these behaviours may signal a change in the motivational state of the animal and good performance seems to be indicative of good wellbeing in animals.[11–15]

Nest building performance has been shown to be sensitive to several diseases and neurological impairments. A decrease in this spontaneous behaviour correlates with brain lesions,[16,17] and genetic mutations,[18] as well as the progression of scrapie and Alzheimer's disease.[19,20] Nest building is compromised by the systemic injection of MPTP, a Parkinson model,[14] and in a model of Rett syndrome.[21] Also, in LPS-treated mice, maternal nest building is decreased significantly.[22]

A previous study from our laboratory revealed a correlation between postsurgical pain and nest building performance in laboratory mice.[12] However, a standardized protocol for the assessment of pain by means of nest building performance has not been developed so far.

In the present study we used 'latency to nest building' and a nest complexity scoring scale similar to already published nest building assessment methods.[1,2,14,23,24]

We analysed the general nest building performance of healthy mice of different strains, sexes and housing conditions to evaluate genetic and environmental influences on nest building.

We assume that successful assessment of nest building performance depends on the right testing time points, as mice tend to destroy and rebuild their nests in a circadian rhythm. Therefore, to determine a suitable testing time we analysed the normal nest building rhythm in healthy C57BL/6J mice.

Using this determined time point, we aimed to prove the feasibility and reliability of nest complexity scoring as a method for detecting mild to moderate postsurgical pain in laboratory mice, and to standardize this method for routine laboratory use. For this purpose, individually-housed female C57BL/6J mice underwent a sham embryo transfer with or without different doses of the analgesic carprofen or underwent control treatment only.

We hypothesized that nest complexity is a sensitive and reliable tool that can be used to assess and grade postsurgical pain in mice.

Materials and methods

Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under licence no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No. 123 Strasbourg 1985) and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Animals

The animals were 48 female and eight male C57BL/6J, eight female DBA/2J, and eight female B6D2-Tg(Pr-mSMalphaActin)V5rCLR-25 mice, obtained from our in-house breeding facility at the age of 6–8 weeks.

The animals' health status was monitored throughout the experiments by a health surveillance programme according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines. The mice were free of all viral, bacterial, and parasitic pathogens listed in the FELASA recommendations, except for *Helicobacter* species.[25]

All the animals were housed in groups of three to six animals for at least three weeks prior to testing in our animal room. The animals were kept in Eurotype III clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding and one nestlet^{TM} (5 cm × 5 cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland) as nest building material. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to sterilized drinking water. The light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 Lux in the cage). The temperature was $21 \pm 1^{\circ}C$, with a relative humidity of $45 \pm 10\%$, and with 15 complete changes of filtered air per hour HEPA H13 filter, Camfil AG, Unterägeri, Switzerland. The animal room was insulated to prevent electronic and other noise. Disturbances, e.g. visitors or unrelated experimental procedures in the animal room, were not allowed.

Experiments

Latency to nest building: effects of strain, sex and housing conditions. Latency to first nest building activity was determined in mice of the three different strains, both sexes and under different housing conditions to analyse effects of these factors on nest building performance.

Eight female and eight male C57BL/6J, eight female DBA/2J, and eight female B6D2-Tg(Pr-mSMalphaActin)

V5rCLR-25 mice, housed individually, and eight pairs of female C57BL/6J mice were tested in their familiar home cages. Additionally, eight female C57BL/6J mice, housed individually, were transferred to a new and clean cage directly before testing.

The mice were housed individually or in pairs three days prior to and during the observations. At the start of the 3-day adaptation phase, one nestlet was placed in the cage. Prior to observation at the beginning of the light phase, nestlet material was removed and a new nestlet was placed in the cage at the beginning of the light phase. Animals were video recorded for 24 h with an infrared-sensitive camera fixed above the cage.

All video recordings were analysed with ObserverXT^{TM} 9 software (Noldus, Wageningen, The Netherlands). Nest building activity was defined as manipulating or carrying the nestlet or nestlet material for more than 3 s, and the latency to nest building was recorded in seconds.

Assessment of circadian nest building rhythm. The 24 h observations of eight individually-housed female C57BL/6J mice in their familiar home cage (the same mice as used in the assessment of latency to nest building) were analysed to assess normal circadian nest building rhythms in order to determine the optimal time point for nest complexity scoring.

All video recordings were analysed with ObserverXT^{TM} 9 software, and nest building duration in seconds was recorded continuously. The remaining behaviours were separated into resting (inactive) and other activities and measured in seconds. Additionally nest complexity was scored by carefully approaching the cage without disturbing the animal at eleven time points using the scale described in Figure 1.

Pain assessment with nest complexity scoring. Experimental design: Forty-eight individually-housed female C57BL/6J mice were tested (partly the same mice as used in the previous observations). Animals were tested before (baseline) and after an experimental procedure (experimental). Eight mice were allocated randomly to one of six experimental groups: (1) surgery + anaesthesia (mice underwent anaesthesia and surgery without analgesic treatment), (2) surgery + anaesthesia + low dose analgesia (mice underwent anaesthesia and surgery with 5 mg/kg carprofen), (3) surgery + anaesthesia + high dose analgesia (mice underwent anaesthesia and surgery with 50 mg/kg carprofen), (4) anaesthesia only, (5) anaesthesia + low dose analgesia, (6) anaesthesia + high dose analgesia.

Baseline data acquisition: Mice were housed individually three days prior to and during testing. At the start of the 3-day adaptation phase, one nestlet was placed in the cage. Prior to testing, the nestlet material was removed and a new nestlet was placed in the home cage at the beginning of the light phase.

Nest scoring (Figure 1) was carried out in the animal room by blinded observers 9 h after providing the nestlet, as this was found to be the optimal time point for nest complexity scoring in the analysis of circadian nest building rhythm (see also Results).

Experiments and experimental data acquisition: Experimental scoring of nest complexity was performed 2 days after baseline measurements. The experiment began at 1.5 h before the start of the light phase with a subcutaneous injection of 2 μL/g body weight of phosphate buffered saline (PBS) for the surgery + anaesthesia and anaesthesia-only groups. In the surgery + anaesthesia + analgesia and anaesthesia + analgesia groups, 5 or 50 mg/kg body weight of the analgesic carprofen (Rimadyl^{TM}, Pfizer Inc, New York, NY, USA) was diluted in PBS and injected subcutaneously as 2 μL/g body weight. Forty-five minutes later, the animals were transferred in individual transport cages to the operating theatre, which was located nearby. Mice were anaesthetized with sevoflurane (Sevorane^{TM}, Abbott, Baar, Switzerland) as a mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen was used as a carrier gas. After induction of anaesthesia in a Perspex induction chamber (8% sevoflurane, 600 mL/min gas flow) animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 39 ± 1°C to ensure constant body temperature, and anaesthesia was maintained via a nose mask (6–7% sevoflurane, 600 mL/min gas flow). The fur was clipped and the operating field disinfected with ethanol in all animals. Mice of surgery groups underwent a one-side sham embryo transfer. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl^{TM}, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany) and the skin was closed using skin staples (Precise^{TM}, 3 M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in the surgery groups. Anaesthesia lasted 14–16 min in all groups. Animals were allowed to recover for 15–20 min on the warming mat before being transferred back to the animal room for subsequent behavioural testing.

The testing began at the beginning of the light phase after removing the used nestlet and adding a new nestlet by returning each mouse from its transport cage to its home cage. At 9 h after providing the new nestlet, nest scoring was carried out by carefully approaching the cage without disturbing the animal.

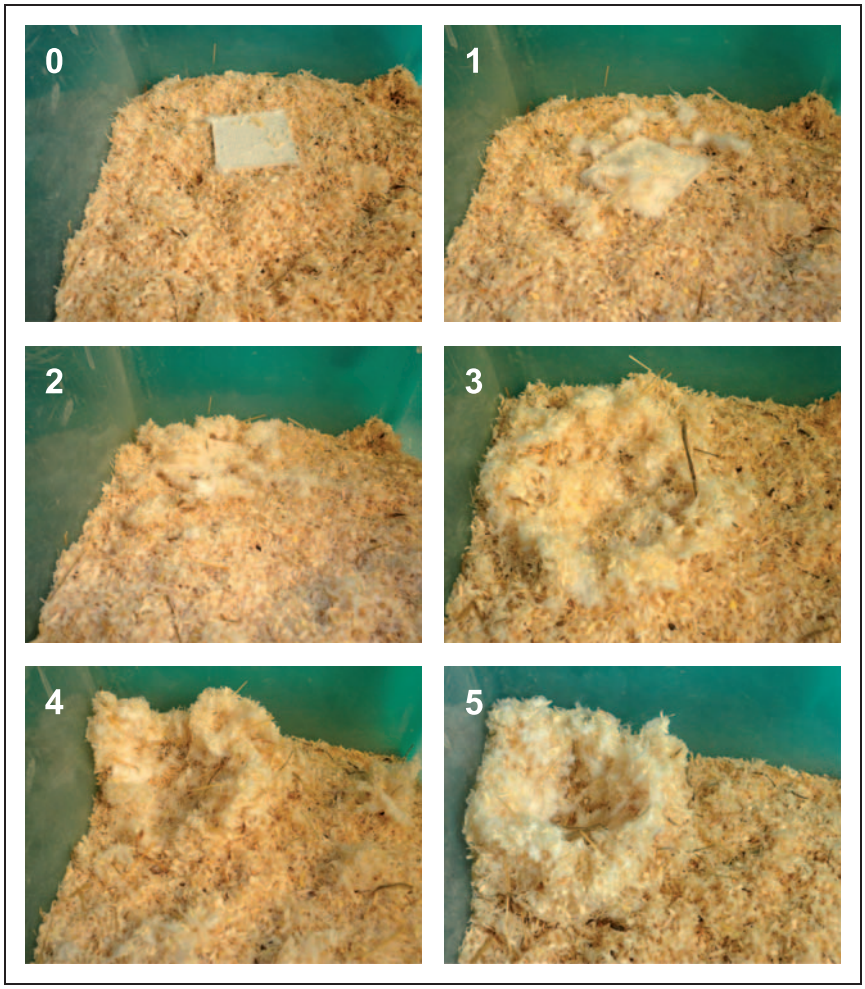

Figure 1. Nest complexity scoring: Score 0 = nestlet not manipulated, possibly dragged around the cage; Score 1 = nestlet slightly manipulated, more than 80% of nestlet intact, possibly a few shreds picked out; Score 2 = nestlet noticeably manipulated, less than 80% of nestlet intact, shreds spread around or in one area; Score 3 = noticeable nest site; less than 80% of nestlet intact, shreds are placed mostly in the nest site, hollow in bedding, mice start building walls; Score 4 = flat nest, hollow in bedding, walls mainly higher than mice and encasing the nest less than 50%; Score 5 = complex nest, more than 50% shreds picked out, bowl-shaped nest, walls higher than mice and encasing the nest by more than 50%.

Statistical data analysis

Statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA). All data were tested for normal distribution and homogeneity of variance and met the necessary assumptions for parametric analyses. Mean and standard error of the mean (SEM) of latency to nest building, duration of nest building, and nest complexity scores for baseline and experimental measurements were calculated. Latencies to nest building were compared between different strains or housing conditions with a one-way analysis of variance (ANOVA). To compare the effect of sex on latency to nest building, an independent two-sample *t*-test was used. To test for significant differences between nest scores general linear model for repeated measures with time as within- and treatment as between-subject factor was used; post hoc testing was conducted with the Bonferroni test. Significance for all statistical tests was established at $P < 0.05$.

Results

Latency to nest building: effects of strain, sex and housing conditions

No significant differences were found in latencies to nest building between strains ($P = 0.415$), sexes ($P = 0.741$), and housing conditions (social environment/physical environment) ($P = 0.871$) (Table 1).

Table 1. Latency to nest building: mean latency (SEM) of all strains, sexes and housing conditions.

Strain	Sex	Housing condition	Mean (min)	SEM (min)
C57BL/6J	Female	Individual housing/familiar cage	60.5	16.5
	Female	Individual housing/new cage	62.5	15.2
	Female	Pair housing/familiar cage	52.5	10.1
	Male	Individual housing/familiar cage	54.6	5.9
DBA/2J	Female	Individual housing/familiar cage	44.3	6.6
B6D2-Tg(PrmSMalphaActin) V5rCLR-25	Female	Individual housing/familiar cage	37.9	11.2

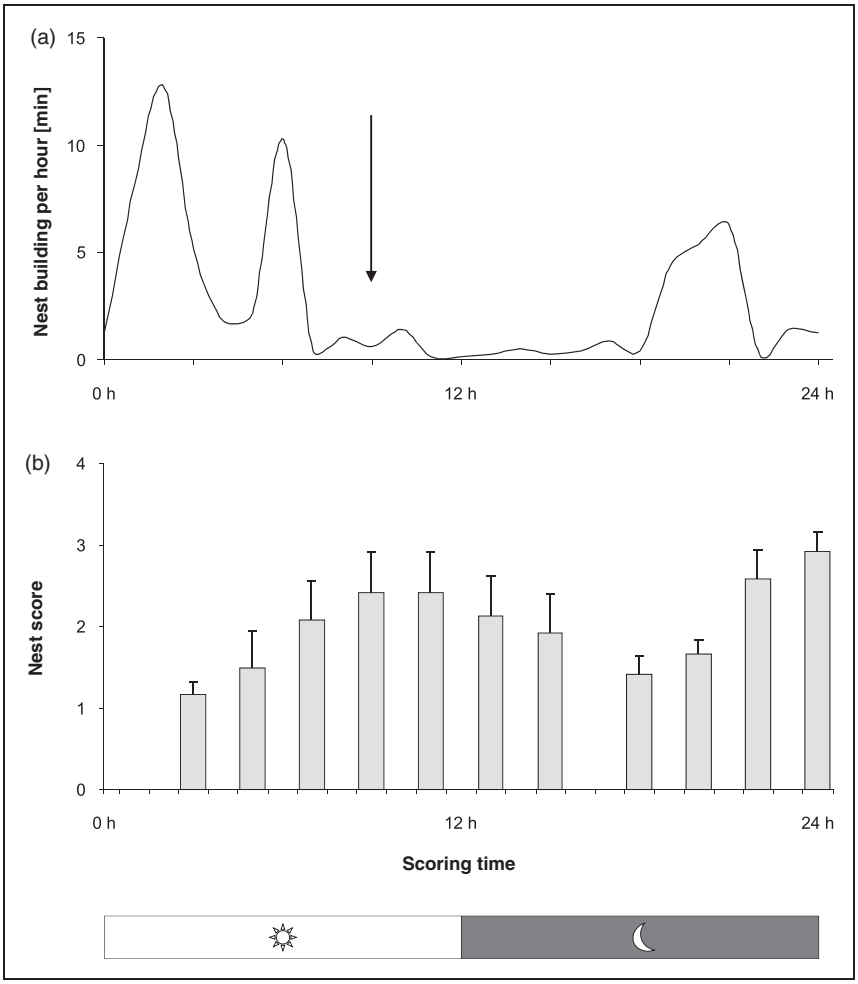

Figure 2. (a) Normal nest building activity during 24 h in female individually-housed C57BL/6J mice. Mean duration of nest building in minutes per observed hour is shown on the vertical axis. (b) Determination of optimal scoring time: Mean nest scores (+SEM) at 11 scoring time points. Scores increased during light phase, decreased at the onset of activity during the dark phase and increased towards a maximum at the end of the dark phase.
Time point of nest complexity scoring for pain assessment is indicated with arrow (9 h after start of the light phase).

Circadian nest building rhythm

Mice were occupied with nest building for, on average, 62.3 (SEM 22.6) min during 24 h of observation, which is equivalent to 4.3% of the day.

On average, mice started with nest building within the first 2 h after nest material was provided. This phase with high nest building activity (Figure 2a) was followed by resting phases with short disruptions for several behavioural activities and short nest building

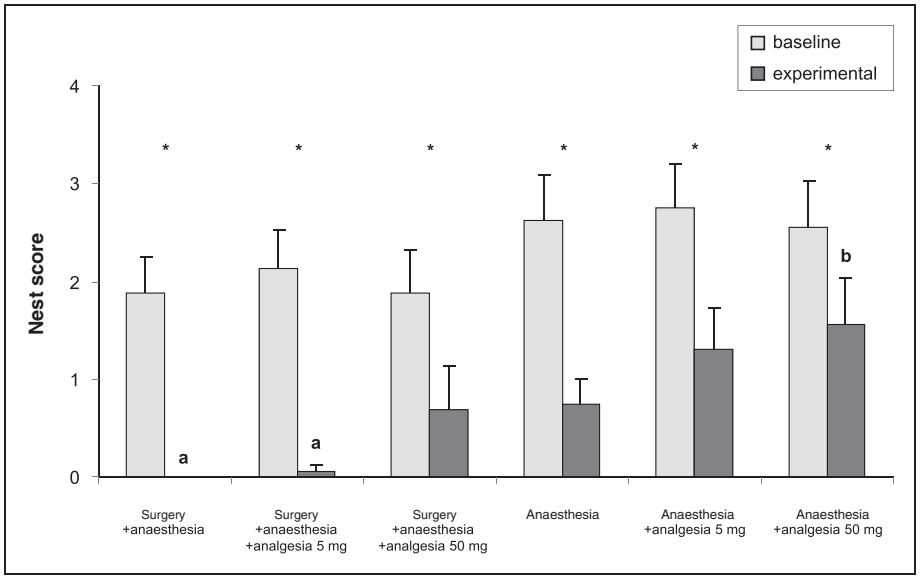

Figure 3. Mean nest complexity scores (+SEM) of female individually-housed C57BL/6J mice at 9 h. Baseline and experimental measurements of all experimental groups are shown. Asterisks indicate within-subject effect of time (baseline versus experimental) at $P < 0.001$. A significant between-subject effect of treatment was found ($P < 0.05$). Different letters over the bars indicate the differences revealed by post hoc analysis between nest scores of experimental groups at $P < 0.05$ (a versus b).

periods for rebuilding or maintaining the nest. In this late light phase nest scores reached high values. During the start of the dark phase, the mice normally had periods of locomotor activity with short breaks for occasional resting. In this active phase, the nest was usually destroyed or flattened out in the bedding by running over the nest site or digging in the bedding, which resulted in a decrease of nest complexity. From the middle to the end of the dark phase, mice started to rebuild their nests, interrupted by resting periods. On average, mice showed higher nest building activity towards the end of the dark phase with increasing nest scores.

Regarding the analysis of the video sequences and the nest scoring, a single time point, 9 h after providing the new nestlet and the start of the light phase, was chosen for determination of the nest complexity scores for pain assessment in the following experiments (Figure 2a and b, arrow).

Nest complexity scoring

Nest scores showed a gradation after experiments (Figure 3). While animals that underwent surgery without or with a low dose of analgesia did not construct noticeable nests, animals treated with higher doses of analgesia had nest scores comparable with anaesthesia-only animals. Animals that received anaesthesia and analgesic treatment only had the highest experimental nest scores.

A significant within-subject effect of time (baseline versus experimental, $P < 0.001$) as well as a significant between-subjects effect of treatments ($P = 0.024$), but no interactions between time and treatment were found. Differences between nest scores of experimental groups were significant when comparing surgery + anaesthesia and surgery + anaesthesia + analgesia 5 mg with anaesthesia + analgesia 50 mg ($P = 0.022$; $P = 0.031$; Figure 3).

Overall, in baseline measurements 75–88% of all mice had noticeable-to-complex nests or at least their cages showed signs of noticeable nestlet manipulation, i.e. scores of two and higher, at the defined scoring time. After the experiments, the percentage of animals with noticeably manipulated nestlets and/or noticeable nests decreased to less than 63%, while animals after surgery without or with a low dose of analgesia never had noticeable nests and only less than 10% of these animals showed noticeable nestlet manipulation (Figure 4).

Discussion

All healthy mice investigated in this study exhibited complex nest building behaviour and constructed nests regardless of strain or sex and under all housing conditions tested. This situation changed distinctly when animals underwent a surgical or anaesthetic procedure: an incremental decrease of nest building performance was observed, correlating with the degree of invasiveness of the experiment.

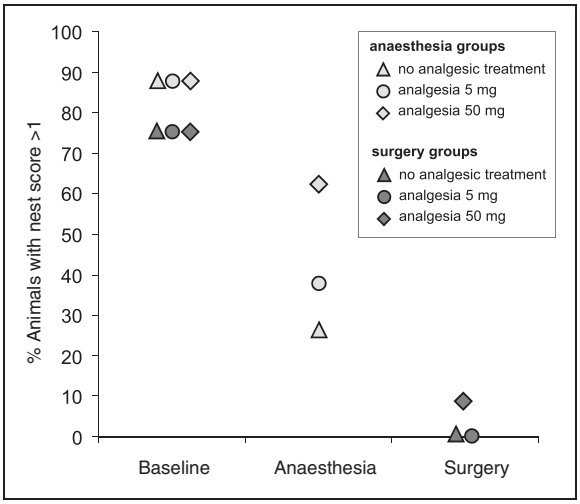

Figure 4. Percentage of female individually housed C57BL/6J mice of different experimental groups with noticeably manipulated nestlet and/or noticeable nests (nest complexity score >1) before and after experimental treatment at 9 hours at the end of the light phase. After experiments (i.) < 10% animals with noticeably manipulated nestlet and/or noticeable nests were found in surgery groups, (ii.) 20 - 60% animals in anaesthesia groups, (iii.) and >75% animals in baseline groups (healthy mice).

We propose that changes in the highly motivated nest building behaviour can be used as a robust indicator of reduced animal wellbeing as also suggested previously by Arras et al.[12] and Deacon[2]. To establish a standardized protocol for the evaluation of postsurgical pain by nest building behaviour, we analysed common factors that could impact on the measurements and consequently influence the transferability of the monitoring protocol. Variability between sexes or genotypes regarding the amount of nest material used (e.g. weight of cotton fibres) or the shape and complexity of the nests have been reported.[4,26] However, in our study, the motivation to use the offered nest building material seemed to be high, and was comparable in different strains, sexes and under the different housing conditions used, as no difference in latency to nest building was observed in healthy mice. Most mice began to manipulate the nest material rapidly and spontaneously within minutes after the material was placed in the cage. Although small inter-individual differences in the shape and complexity of the nests appeared, individuals appeared to be consistent – an observation also described by others.[4]

We then tested the feasibility of nest complexity scoring in a set-up, with female C57BL/6J mice housed individually in their familiar home cages. Since we observed in our daily work that mice in general destroyed their nests during locomotor activity periods, we considered the appropriate choice of testing time to be an important prerequisite for successful complexity scoring. Nest complexity scoring is often done in the morning after material having been provided the day before (see Ref 2 for example), but surgery or other experimental procedures are often conducted during the day, and pain or other impairment are likely to be most significant directly after such procedures. In experiments with minor invasiveness, it is possible that nest scoring 24 h post procedure might miss the most pronounced signs of reduced wellbeing, as nest scores seemed to increase in our study during the dark phase (i.e. at 22 and 24 h after the experiment) up to baseline values (data not shown). Therefore we aimed to identify the appropriate testing time, considering the time point of the experiment and adapting to laboratory routine schedules.

The results of our analyses of behavioural circadian rhythmicity were comparable with the findings of other studies, showing that mice normally build a new nest or repair an old nest at the end of the dark phase.[27,28] Nest building peaks of our mice were shifted towards the beginning of the light phase, which was perhaps associated with the fact that fresh nest building material was provided in this phase. After pronounced nest building activity, animals rested in their nests and nest complexity scores were high and remained relatively constant in this phase. Additionally, this phase with constant nest scores fell within the normal working hours of laboratory personnel, which is a relevant argument for a method that should be applicable under routine laboratory conditions.

Based on these data, we chose a scoring time point of 9 h after experimental treatments at the end of the light phase. However, several hours before this time point also appear to be suitable for successful nest complexity scoring. By this means, nest scoring can act as a short-term retrospective indicator of impairment, which can be applied easily within the normal working day, particularly if procedures are conducted in the early morning. Thus, mice that have suffered, or are still suffering because of inefficient analgesic treatment can be identified easily and can be provided with rescue analgesic treatment.

A total of 75–88% of all healthy mice had identifiable nests or cages that showed at least noticeable nestlet manipulation leading to mean nest scores of 2–3. The maximum scores of 4–5 were difficult to reach within 9 h – in particular for a single-housed mouse – as the nestlet was a quadrate of tightly packed cotton fibres which thus needed intense work to reconstitute into a nest. Therefore we assume that nest scores of two and higher are normal nest scores for healthy individually housed mice after 9 hours.

In contrast to the substantial nest building performance of healthy mice, i.e. nest scores of 2 and above

(see Figure 4), none of the animals undergoing surgery had noticeable nests 9 h after experiments and less than 10% of the animals manipulated the nestlet noticeably, suggesting that these groups may suffer from distress, impaired general condition or even pain.

As observed already in previous studies,[12,13,29] anaesthesia alone had a marked impact on behaviour. Here we observed a distinct effect of anaesthesia on nest building behaviour, which was significantly relative to the baseline, but clearly did not affect the animals as strongly as surgery, indicating only mild impairment. Animals that underwent anaesthesia and additional carprofen treatment of different doses had higher nest scores than anaesthesia-only groups. Whether carprofen can inhibit anaesthesia-induced behavioural aberrations, and thus have a nest building promoting effect, could not be clarified in this study. To our knowledge no behavioural effect of carprofen has been described that might explain these results.

Animals that received the low dose analgesic carprofen before surgery did not show a clear increase in nest complexity, which might be a sign that this dose was too low to relieve postsurgical pain, despite the fact that 5 mg/kg is a standard dose for mice[30] and has been shown to act as an effective analgesic protocol after surgery.[13] In the higher dose (50 mg/kg), we observed a slight tendency towards higher nest complexity comparable with the anaesthesia-only group, although not as high as in anaesthesia with analgesia groups.

As the clear and significant difference in nest complexity between healthy mice and mice that underwent surgery could not be alleviated by carprofen treatment in a significant manner, our study lacked a sound proof that postsurgical pain caused the massive decrease in nest complexity. Thus, other impacts of surgery might also affect nest complexity, e.g. physiological stress or motor impairment. However, it is very unlikely that carprofen in the used dose rates was not capable of pain relief as it has been proved to be effective after laparotomy in several studies (see Refs 13 and 31 for examples). Nest complexity scoring might therefore be a useful indicator of reduced wellbeing after surgery, but cannot be used to assess the efficacy of pain treatment. Nevertheless, the estimation, i.e. grading, of the impact of procedures on wellbeing and general condition is possible with nest complexity scoring.

Our results suggest that deficits of nest building are associated with reduced wellbeing and impaired general condition,[12] which can also include pain, and may trigger a competitive motivational system that makes the animal tend to be lethargic or be concerned with other behaviours like self grooming and with decreased motivation to engage in otherwise highly valued nest building behaviour.[22,32]

Nest complexity scoring is based on the animal's normal behaviour performed in the animal's home cage, and does not require special apparatus or housing facilities. The test causes no additional stress to the animals, as nest building is a species-specific and complex form of active interaction with the environment. Providing nest material allows mice to structure their environment and gain more control over their living conditions, which is assumed to enhance their wellbeing.[10,20,33,34]

In summary, nest complexity scoring can be implemented easily in any laboratory animal facility and can be applied in the daily routine for the detection and assessment of post-procedural impairment in laboratory mice. Even though the motivation to use the nest material was comparable under different conditions, it might be necessary to adapt the scoring system to other nest building material or housing conditions. As social housing is the preferred housing condition for mice and has been suggested to enhance postsurgical recovery in female mice,[15,29,35] further studies should focus on an adaption of the described assessment method to prevalent housing conditions like pair or group housing.

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Chapter 5: Individual housing of female mice: influence on post-surgical behaviour and recovery

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Original Article

Individual housing of female mice: influence on postsurgical behaviour and recovery

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Abstract

Individual housing of laboratory mice may increase vulnerability to surgical stress, and interfere with postsurgical recovery. To analyse the effect of housing conditions on recovery, pair- and single-housed female C57BL/6J mice underwent a minor laparotomy +/− analgesia, anaesthesia only or no treatment. Animals were monitored using non-invasive methods during the immediate postsurgical period to assess pain and general impairment. While no appearance or posture abnormalities were observed postexperiment, home cage behaviours were affected distinctly. Discriminant analysis identified self-grooming, locomotion, climbing and resting as mainly responsible for experimental group separation. Behavioural rhythmicity was disrupted, and behaviours related to wellbeing, such as nest building, climbing and burrowing, decreased. Behavioural pain signs (e.g. press) increased. Most behavioural alterations showed a gradation between treatments, e.g. burrowing latency ranged from an intermediate level following anaesthesia only and surgery with analgesia, to pronounced prolongation after surgery without analgesia. Significantly lower burrowing performance after surgery without analgesia in individually-housed animals indicates better recovery in pairs. Social interaction in pairs – an important component of normal behaviour (64%) and a potential indicator for direct social support – was nearly absent (0.3–0.5%). While anaesthesia and surgery resulted in clear changes in behaviour, differences between housing conditions were minor. Hence, despite a tendency towards better recovery in pairs, we found no distinct negative effect of individual housing. In conclusion, both housing conditions are acceptable during the period immediately following minor surgery, though social housing is always preferable in female mice.

Keywords: Mice, postsurgical recovery, behaviour, individual housing, refinement

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Laboratory mice, just like their wild ancestors, are social animals and are highly motivated to interact with each other.[1] A stable and harmonious social context seems to be important for mouse wellbeing. While providing harmonious groups may be difficult in sometimes aggressive male mice, the advantage of housing female mice in groups is clear since the environment in which animals are housed influences not only animal wellbeing but also experimental results.[1] Despite the common practice of social housing in animal facilities, mice are often separated for scientific or practical reasons such as for monitoring purposes or surgical procedures.

The findings of studies analysing the actual effects on scientific results of individual housing of mice, and the possible adverse impact on wellbeing, have been ambiguous. Some studies found no effect of individual housing of different duration compared with social housing on endocrine stress indicators,[2] physiological indexes[3] or behavioural tests.[2] On the contrary, several other studies have shown distinct effects of individual housing compared with social housing, as evidenced by changes in sympathetic neurotransmission,[4] basal heart rate[5,6] and thymus weight.[6] Other alterations are disruptions to circadian activity patterns[5] and effects on memory, emotionality and anxiety, as well as a tendency to show hyperactivity in behavioural tests.[7–9]

Although the proximate effects of individual housing may not always be evident, it might affect the way animals respond to stressors. Even if normal behaviour and general condition are unaffected, some studies have shown that individually-housed or isolated animals were more sensitive to stress, with mice housed in stable groups recovering faster

from mild stressors,[6,10] leading to the suggestion that individual housing may hamper the animal's ability to cope with, and increase vulnerability to, stressful episodes.

Surgery and the postsurgical recovery phase represent stressful episodes for mice. Hence, individual housing may exacerbate an animal's vulnerability to surgical stress and may interfere with postsurgical recovery. Two studies seem to support the hypothesis that social housing has a recovery-promoting effect. Pham *et al.*[11] found that, after laparotomy and caecal manipulation, single-housed mice exhibited greater self-administration of analgesics than social-housed mice and therefore seemed to experience more pain postsurgery. In another study, faster recovery was observed in socially-housed mice following major surgery.[12]

Here, we aimed to analyse the potentially beneficial effect of social support on postsurgical recovery by comparing single-housed with pair-housed female C57BL/6J mice after minor surgery. To assess the impact of surgery and different housing conditions on wellbeing we used a range of non-invasive behavioural measurements that can be applied in the animals' home cage without provoking additional stress. Pain signs,[13,14] burrowing performance,[15] home cage behaviours[12] and classical indices like clinical symptoms, overall appearance and body weight should allow recognition not only of postsurgical pain but also impairment of general condition, thus providing a broad picture of the animal's recovery.

We hypothesize that signs of pain and impaired wellbeing should be reduced in socially-housed mice if this housing condition is beneficial to postsurgical recovery.

Materials and methods

Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under licence no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to the *European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes* (Council of Europe no. 123 Strasbourg 1985) and to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Animals

Sixty-four C57BL/6J and 32 C57BL/6J-Tyr$^{C\text{-}Brd}$ female mice were obtained from our in-house breeding facility at the age of 6–8 weeks. We used pairs of black-coated C57BL/6J strain and its coisogenic albino mutant C57BL/6J-Tyr$^{C\text{-}Brd}$ for better optical distinction of the observed animals. The mice were free of all viral, bacterial and parasitic pathogens listed in the FELASA recommendations. Health status was confirmed by a health surveillance programme throughout the experiments.[16]

Mice were housed in groups of three to six animals for at least three weeks prior to testing in our animal room. Animals were kept in Eurostandard Type III clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding (80–90 g per cage) and one nestlet^{TM} (5 cm × 5 cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland) as nesting material. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) *ad libitum* (provided in a food hopper continuously throughout the entire duration of the experiment) and had unrestricted access to sterilized drinking water. The light/dark cycle in the room consisted of 12/12 h (lights on 15:00, lights off 03:00) with artificial light (approximately 40 lx in the cage). The temperature was 21 ± 1°C, with a relative humidity of 50 ± 5%, and with 15 complete changes of filtered air per hour (HEPA H 14 filter). The animal room was insulated to prevent electronic and other noise. Disturbances, e.g. visitors or unrelated experimental procedures in the animal room, were not allowed.

Experiments

Experimental housing and set-up

During the whole experimental period animals were housed under standardized conditions as described above with the burrowing test set-up in addition. As burrowing apparatus, a plastic bottle (standard opaque water bottle, 250 mL, 150 mm length, 55 mm diameter) filled with 138–142 g of food pellets identical to those of the animal's normal diet was used. An additional empty bottle of the same dimensions was provided to serve as a shelter.

Experimental design

Mice were housed either in pairs of one C57BL/6J and one C57BL/6J-Tyr$^{C\text{-}Brd}$ mouse, or individually (one C57BL/6J). Each pair- or single-housed mouse was observed directly after an experimental procedure. Eight pairs and eight individually-housed mice were allocated randomly to one of four experimental groups: (1) surgery + anaesthesia (mice underwent anaesthesia and surgery without analgesic treatment); (2) surgery + anaesthesia + analgesia (mice underwent anaesthesia and surgery with analgesic treatment), (3) anaesthesia only or (4) no treatment.

Experiments and data acquisition

For acclimatization, animals were housed either in pairs or individually for three days under standardized conditions in cages containing the experimental set-up as described above. Animals had no prior experience with behavioural testing.

In pair-housed mice, both animals underwent experimental procedures. The experiment began at 13:00 h with a subcutaneous injection of 2 μL/g body weight of phosphate-buffered saline (PBS) for the surgery + anaesthesia and anaesthesia-only groups. In the surgery + anaesthesia + analgesia group, 5 mg/kg body weight of the analgesic carprofen (Rimadyl^{TM}, Pfizer Inc, New York, NY, USA) was diluted in PBS and injected as 2 μL/g body weight. The animals were transferred 45 min later in transport cages to the nearby operating theatre. Mice were anaesthetized with sevoflurane (Sevorane^{TM}, Abbott, Baar, Switzerland) as

mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen was used as carrier gas. After induction of anaesthesia in a Perspex induction chamber (8% sevoflurane, 600 mL/min gas flow), animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at $39 \pm 1^{\circ}C$ to ensure constant body temperature, and anaesthesia was maintained via a nose mask (4.9% sevoflurane, 600 mL/min gas flow). Eye ointment was applied, the fur was clipped and the operating field disinfected with ethanol (70%) in all animals. Mice in both surgery groups underwent a one-side sham embryo transfer. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl™, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany), and the skin was closed using skin staples (Precise™, 3M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in both surgery groups. Anaesthesia lasted 14–16 min in all groups. Animals were allowed to recover for 15–20 min on the warming mat before being transferred back to the animal room for subsequent behavioural observation.

Observation began at 15:00 h by returning each mouse from its transport cage to its home cage containing the re-filled burrowing test apparatus or, in the case of non-treated mice, just refilling the test apparatus and by starting digital video recording.

Data analysis

Home cage behaviours

The recorded video sequences were analysed using ObserverXT™ software (Noldus, Wageningen, The Netherlands) for the first 6 h of the light phase. Durations of individual behaviours (individual resting, locomotion, self-grooming, digging, eating, drinking, climbing, burrowing, nest building) and numbers of resting bouts were measured. For pairs, durations of social behaviours (social investigation, social grooming, social resting, aggressive behaviour and submissive/subdominant behaviour) were also measured (Table 1[17]). General activity was calculated by summarizing all active behaviours (i.e. all home cage behaviours except resting). Non-defined behaviours were not recorded.

Pain signs

The number of aberrant behaviours occurring in pain states, e.g. press, stretch or stagger/fall and rear up (Table 1[13,14]), was assessed for 15 min at one hour after the start of observation.[18]

Burrowing performance

The burrowing test determines burrowing performance and can be used as a simple method to assess postsurgical impairment in mice. Good performance in this test is defined as short latency to remove items from a tube-like apparatus (burrowing).[15] Burrowing was defined as the removal of more than three pellets from the apparatus within 10 s. The latency to burrow per cage was measured. Measurement of latency was continued for 24 h if the animals did not start to burrow within the 6 h of behavioural analysis.

Table 1 Ethogram of home cage behaviours and pain signs

Individual behaviours	
Individual resting	Sitting or lying flat or curled up, sometimes with the eyes closed or nearly closed (includes sleeping)
Locomotion	Walking, running, jumping
Self-grooming	Bouts of wiping, licking and nibbling their fur with forepaws and tongue
Digging	Removing, or apparently trying to remove, substrate material from a certain place (not the burrowing apparatus); series of fast movements of the fore and/or hind paws
Eating	Series of movements resulting in ingesting food
Drinking	Taking in liquids with series of licking movements of the tongue
Climbing	Climbing with all four feet at the cage grid
Burrowing	All behaviours linked with emptying the burrowing apparatus (digging, carrying, etc. of material)
Nest building	All behaviours linked with nest building (arranging, pulling in, fraying, etc.)
Social behaviours	
Social investigation	Non-aggressive investigation of another individual; attending, approaching, anogenital sniffing, nose sniffing and body sniffing; following the partner without fast or sudden movements
Social grooming	Active animal licking and combing the fur of the partner; grooming animal often leans on his partner with forepaws; passive animal is lying flat, relaxed while the partner performs social grooming
Social resting	Resting together in close body contact
Aggressive behaviour	Includes biting, chasing, pinning, mounting, boxing, attack, threat postures (offensive upright); aggressive grooming
Submissive/ subdominant behaviour	Fleeing, retreating, freezing, submissive postures (defensive upright = sitting upright, head up, forepaws stretched), crouching = lying still, rigid, ears down, eyes closed
Pain signs	
Press	Abdomen pushed to floor
Stretch	Abdomen pushed to floor, hind paws stretched backwards
Stagger/fall	Partial loss of balance
Rear up	Standing on rear legs

Clinical investigation

Animals were weighed at 15:00 h, 24 h before, and 24 and 48 h after experiment and observed for 20–30 s before, during and after weighing. According to a routinely used scoring system documenting the general condition of an animal,[19] abnormalities of body condition (e.g. sunken flanks), fur condition (e.g. ruffled coat), eyes (e.g. discharge), breathing (e.g. irregular) and posture (e.g. hunched back) were registered, and wound healing, spontaneous behaviour and movement were assessed.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 software (IBM, Armonk, NY, USA).

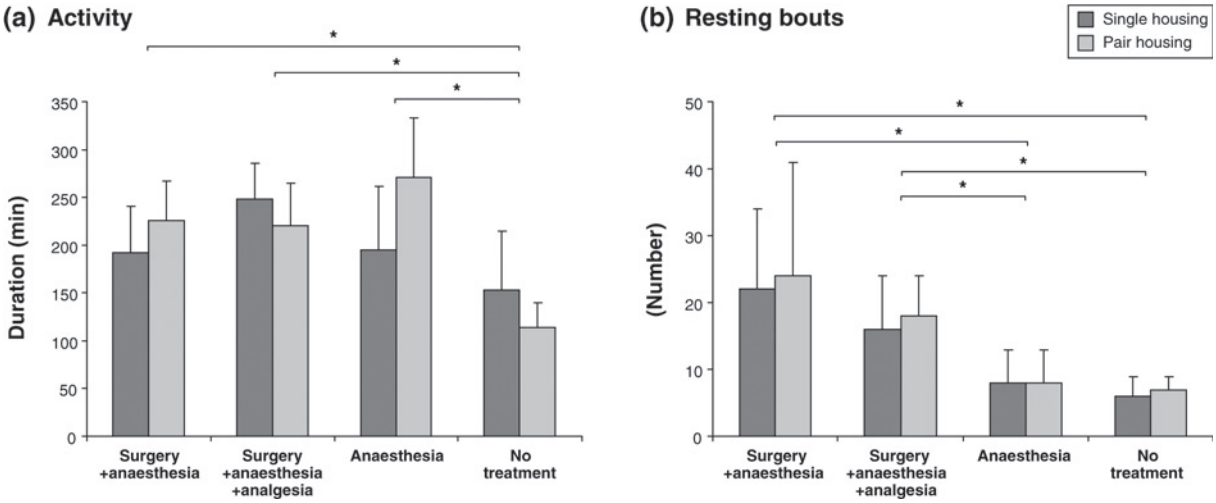

Figure 1 (a) Mean (±SD) duration of general activity. Significant differences were found between the no treatment group and all treated groups ($P < 0.001$). (b) Mean (±SD) number of resting bouts. For pairs, results of individual and social resting were combined. Significant differences were found between the no treatment groups and both surgery groups ($P < 0.001$; $P = 0.009$) and anaesthesia-only animals and surgery groups ($P < 0.001$, $P = 0.036$). Significant results are marked with * ($P \leq 0.05$)

All data were tested for normal distribution and homogeneity of variance. If necessary, data were log $(X + 1)$ transformed to meet assumptions of statistical tests.

Mean and standard deviation (SD) of latency to burrow were calculated. Kaplan–Meier survival analysis was performed to examine the distribution of time to effect (latency to burrow). To test whether latency to burrow differed statistically between experimental groups or housing conditions, a log rank significance test was performed.

Mean and SD of durations of home cage behaviours, numbers of resting bouts and pain signs were calculated. In pair-housed mice, data of only one mouse (i.e. the black coated C57BL/6J) were used for further statistical analysis.

Discriminant analysis was used to determine the effects of surgery, anaesthesia and analgesic treatment on individual and social home cage behaviour; behaviours mainly responsible for group separation were determined. The determined behaviours were further analysed using univariate general linear model (GLM) with experimental group and housing as fixed factors for individual behaviours and experimental group for social behaviours as fixed factor. *Post hoc* tests (Bonferroni) were used for comparisons between experimental groups.

Activity duration and number of resting bouts were compared between groups using a univariate GLM with experimental group and housing as fixed factors. *Post hoc* tests (Bonferroni) were used for comparisons between experimental groups.

Significance for all statistical tests was established at $P \leq 0.05$.

Results

Home cage behaviours

General activity

General activity, determined as the sum of all active behaviours, i.e. all home cage behaviours except resting, was significantly higher in experimental groups in both housing conditions that underwent surgery or anaesthesia compared with no treatment ($P < 0.001$) (Figure 1a, Table 2). Additionally, the interaction between housing and experimental group was significant ($P = 0.006$), while housing condition alone was not a significant factor ($P = 0.391$).

Activity rhythm

The number and temporal distribution of active and passive behaviour bouts was defined as the activity rhythm. In comparison with no treatment, activity rhythms in both housing conditions appeared disrupted following experiments (Figure 2). Non-treated animals, as well as animals that underwent anaesthesia only, showed long resting bouts, disrupted by a few short activity bouts. After surgery, the activity rhythms were highly fragmented into short activity and resting bouts; consequently, the number of resting bouts increased (Figures 1b and 2; Table 2). These differences were significant between no treatment groups and both surgery groups ($P < 0.001$, $P = 0.009$), and between the anaesthesia-only and both surgery groups ($P < 0.001$, $P = 0.036$). Housing condition and interactions between housing and experimental groups were non-significant factors ($P = 0.611$, $P = 0.952$).

Individual behaviours

In comparison with no treatment, overall resting (for pairs, results of individual and social resting were combined), climbing, burrowing and nest building behaviour decreased, while eating and self-grooming behaviour increased in both housing conditions. Locomotion was equal to, or increased compared with, that in the no treatment group but decreased in the surgery + anaesthesia + analgesia groups. Drinking duration was equal in the surgery + anaesthesia groups while it was increased in the other groups under both housing conditions (Table 2).

Table 2 Home cage behaviours

	Single housing				Pair housing			
	Surgery + anaesthesia	Surgery + anaesthesia + analgesia	Anaesthesia	No treatment	Surgery + anaesthesia	Surgery + anaesthesia + analgesia	Anaesthesia	No treatment
Individual behaviours (mean ± SD)								
Resting bouts (*n*)	22 ± 12	16 ± 8	8 ± 5	6 ± 3	24 ± 17	18 ± 6	8 ± 5	7 ± 2
General activity (min)	192 ± 49	248 ± 38	195 ± 66	153 ± 62	226 ± 41	220 ± 45	271 ± 62	114 ± 26
Individual resting (min)	173 ± 50	114 ± 40	171 ± 69	211 ± 55	143 ± 45	143 ± 43	98 ± 60	21 ± 35
Locomotion (min)	49 ± 27	36 ± 14	56 ± 37	51 ± 37	104 ± 72	35 ± 22	108 ± 68	42 ± 12
Self-grooming (min)	121 ± 54	180 ± 31	106 ± 50	61 ± 15	86 ± 72	159 ± 25	57 ± 77	22 ± 15
Digging (min)	4 ± 4	3 ± 5	3 ± 3	4 ± 9	9 ± 11	3 ± 5	10 ± 10	1 ± 1
Eating (min)	9 ± 10	25 ± 17	19 ± 18	9 ± 9	8 ± 4	8 ± 7	9 ± 8	5 ± 3
Drinking (min)	1 ± 1	3 ± 3	2 ± 1	1 ± 1	1 ± 1	1 ± 1	3 ± 2	1 ± 0
Climbing (min)	1 ± 1	1 ± 1	1 ± 2	14 ± 24	11 ± 21	1 ± 1	2 ± 2	22 ± 15
Burrowing (min)	2 ± 4	1 ± 2	3 ± 4	6 ± 2	2 ± 4	1 ± 2	4 ± 4	4 ± 4
Nest building (min)	4 ± 6	1 ± 1	3 ± 4	6 ± 5	5 ± 8	11 ± 7	12 ± 14	16 ± 9
Social behaviours (mean ± SD)								
Social investigation (min)	–	–	–	–	0 ± 0	1 ± 1	0 ± 0	1 ± 1
Social grooming (min)	–	–	–	–	0 ± 0	0 ± 0	0 ± 1	1 ± 1
Social resting (min)	–	–	–	–	0 ± 0	0 ± 0	1 ± 2	226 ± 31
Aggressive behaviour (min)	–	–	–	–	0 ± 0	1 ± 2	0 ± 0	1 ± 1
Submissive/subdominant behaviour (min)	–	–	–	–	0 ± 0	0 ± 0	0 ± 0	1 ± 2

Mean (±SD) duration of individual and social behaviours, general activity and numbers of resting bouts. For resting bouts in pairs results of individual and social resting were combined

Figure 3 shows scatter plots of the discriminant scores generated by the analysis for single- and pair-housed animals. The home cage behaviours analysed contributed to significant group separation. Discriminant analysis of single-housed mouse behaviour found the first function responsible for significant separation of experimental groups (Figure 3a; Wilks' lambda; function 1, $P = 0.010$). This function explained 73.7% of the variance. Overall resting, locomotion and self-grooming made the largest contribution to group separation, but climbing and eating also contributed. In pair-housed mice, the first two functions were responsible for significant separation of the experimental groups (Figure 3b, Wilks' lambda; function 1, $P < 0.001$; function 2, $P = 0.001$). Function 1 explained 71.9% of the variance. Here, self-grooming and locomotion were responsible for group separation. Function 2 contributed 20.8% to separation; here, separation was based on drinking duration. Under both housing conditions, more self-grooming, eating and drinking correlated with anaesthesia or surgery, while resting, nest building, burrowing and climbing were more prevalent in animals that were not treated.

GLM was performed with behaviours that were mainly responsible for experimental group separation to test for significant differences between treatments and housing conditions in these behaviours. Significant differences between no-treatment animals and experimentally-treated animals were found in overall resting (surgery + anaesthesia: $P = 0.001$; surgery + anaesthesia + analgesia: $P < 0.001$; anaesthesia: $P < 0.001$; Figure 4c), climbing (surgery + anaesthesia: $P = 0.041$; surgery + anaesthesia + analgesia: $P = 0.001$; anaesthesia: $P = 0.002$; Figure 4b) and self-grooming (surgery + anaesthesia: $P = 0.003$; surgery + anaesthesia + analgesia: $P < 0.001$; anaesthesia: $P < 0.001$; Figure 4d). Additionally, significant differences were found in locomotion and self-grooming duration between surgery + anaesthesia + analgesia and surgery + anaesthesia as well as anaesthesia-only groups ($P = 0.042$, $P = 0.002$ and $P = 0.015$, $P = 0.011$; Figures 4a and d). Drinking duration was significantly different in animals that underwent anaesthesia only compared with surgery + anaesthesia and non-treated animals ($P = 0.001$, $P = 0.003$). Significant housing effects were found only in locomotion and eating duration ($P = 0.025$, $P = 0.004$). A significant interaction between housing and experimental condition was found in resting and drinking durations ($P = 0.010$, $P = 0.046$).

Social behaviours

In non-treated pair-housed mice, social behaviours occurred often (64% of total observation time); these were mainly socio-positive or socio-neutral behaviours like social grooming and social resting. Social behaviours decreased strongly in experimentally-treated groups (0.3–0.5%) while individual behaviours increased.

When discriminating individual and social resting in pair-housed mice, duration of individual resting was higher in all experimental groups compared with no-treatment animals, while social resting all but disappeared, and overall resting (individual resting + social resting) was lower (Table 2).

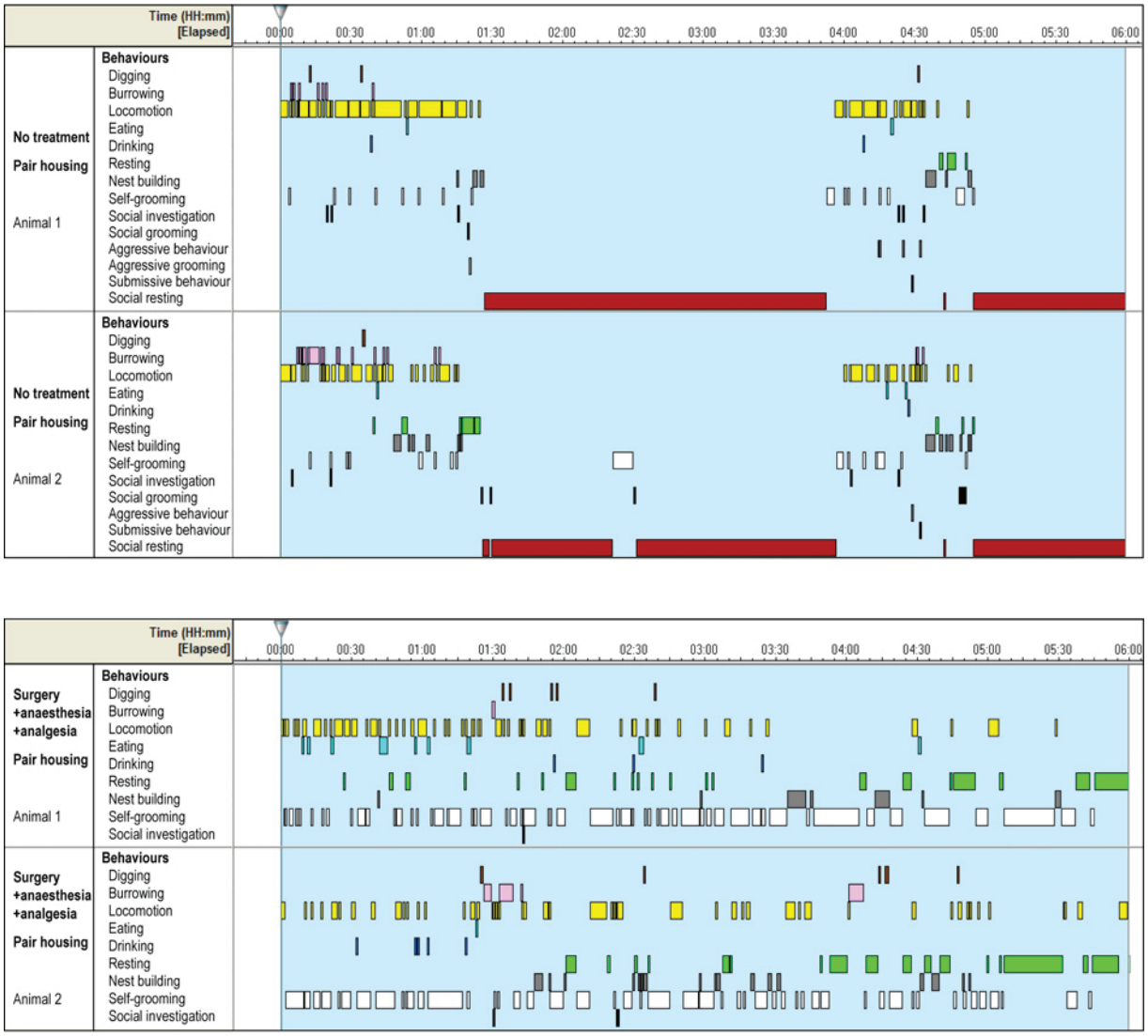

Figure 2 Activity rhythm. Six hours of representative data of pairs of mice that were not treated, and after surgery + anaesthesia + analgesia. Bars represent the occurrence and duration of observed behaviours (for statistical analysis aggressive grooming was integrated with aggressive behaviour). While both animals showed a clear rhythm, occurring in synchrony with each other, after surgery the rhythm was disrupted into short behavioural bouts. Notably, social resting (i.e. animals in body contact during resting) was absent after experiment

In the discriminant analysis of social behaviours, function 1 explained 99.7% (Wilks' lambda; $P < 0.001$) of the variance. Social resting was the only behaviour responsible for group separation and was therefore further analysed with GLM. Social resting duration was significantly longer in non-treated mice compared with other groups (each $P < 0.001$).

Also, the apparent synchronization of activity rhythm in pairs was lost after experiments compared with non-treated mice (see representative example in Figure 2).

Pain signs

While *fall* never occurred during our observations, *press*, *stretch* and *stagger* occurred only in treated animals but never in non-treated animals. These behaviours were very rare, low-frequency incidents and showed a high individual variability that precluded reliable analysis. Even when combining the measurements *press*, *stretch* and *stagger* as a composite scale, no significant difference in the number was found between experimental groups or housing conditions (data not shown). *Rear up* was observed significantly more often in non-treated mice compared with other groups but no significant difference in the number of observations was found between other groups or housing conditions (data not shown).

Burrowing performance

In both housing conditions, surgery without pain treatment resulted in longer latencies than surgery with analgesia, while anaesthesia resulted in the smallest increase in latency compared with no-treatment animals.

Kaplan–Meier analyses showed significant differences between non-treated animals and the experimentally-treated groups under both housing conditions ($P < 0.001$).

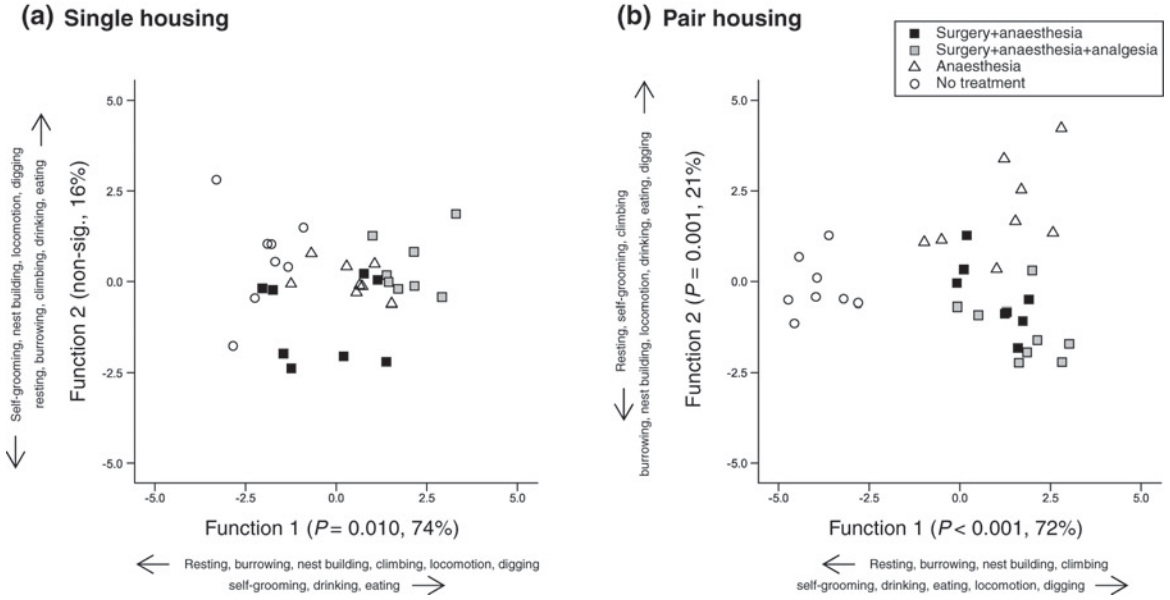

Figure 3 Scatter plot of discriminant scores assigned to individual mice of each housing condition in the different experimental groups: (a) single-housed mice and (b) pair-housed mice. The significance of each function in separating groups, and their percentage contribution to between-group variance are shown on each axis. Additionally, behaviours are displayed together with their correlation with each function (increase or decrease)

Pair-housed mice showed also significant differences when surgery + anaesthesia and surgery + anaesthesia + analgesia groups were compared with the anaesthesia group ($P = 0.03$, $P = 0.014$), but differences were significant in single-housed mice only when comparing surgery + anaesthesia and anaesthesia groups ($P = 0.003$) (Figure 5). Comparing both housing conditions, latencies were shorter for pair-housed mice compared with single-housed mice, with an exception in non-treated animals, which was significant after surgery + anaesthesia and anaesthesia only ($P = 0.050$, $P = 0.021$) (Figure 5).

Clinical investigation

No abnormalities in appearance, posture or spontaneous movements were detected. No complications in wound healing were observed, nor any manipulation of the wound by the cage mate in pair-housed mice. No changes in body weight compared with one day before experiments occurred at either one or two days after experiments (data not shown). Clinical investigation revealed unaltered general condition scores in all groups.

Discussion

This study was set up to determine whether social or individual housing is more beneficial for postsurgical recovery and wellbeing of female mice. For this purpose, animals were monitored closely during the period immediately after surgery. Behavioural investigations revealed only slight tendencies towards better wellbeing in social housing and no adverse effects (e.g. aggression, wound manipulation by cage mate) were detected. However, social interaction, which forms a large part of the behaviour of healthy females and may be an indicator for direct social support, was nearly absent after experiments. As no pronounced detrimental effect of single housing was seen, and differences between single- and pair-housed mice were only minor, no definite conclusion can be drawn that one housing condition was clearly superior.

Clinical investigations, focusing on changes in appearance, posture and body weight, carried out daily are standard monitoring tools after surgery. Since no abnormalities were detected with these investigations, we suggest that our model has only low impact on condition, health and wellbeing, particularly in comparison with other models of surgery (e.g. Ref. 11).

Pain signs occurred only after surgery or anaesthesia and never in non-treated mice but were very variable and infrequent, with one exception. Rear up was observed significantly more in non-treated mice. Since we identified the signs from video recordings of the home cage and not a designated cage with arrangements for visibility,[13,14] some of these subtle signs may have been overlooked. However, since pain may have been less intensive in our model, pain signs may have been exhibited rarely by our animals. Altogether, no differences between treatments or single- and pair-housed mice could be established with these signs.

The rhythmicity and duration of most home cage behaviours, and the latency to burrow differed clearly between treated and non-treated mice, suggesting a decrease in wellbeing following experiments.

Healthy mice mostly rest during the light phase and show a stable circadian rhythm; disruption of this rhythm might indicate impaired wellbeing.[20] In our study, overall resting duration was decreased in treated animals, accompanied by more and shorter resting bouts, resulting in a dramatic disruption of the activity rhythm in all surgery groups, while anaesthesia alone had no pronounced effects on rhythmicity.

Nevertheless, the consequent increase in general activity, mainly due to self-grooming, seemed greater in mice that

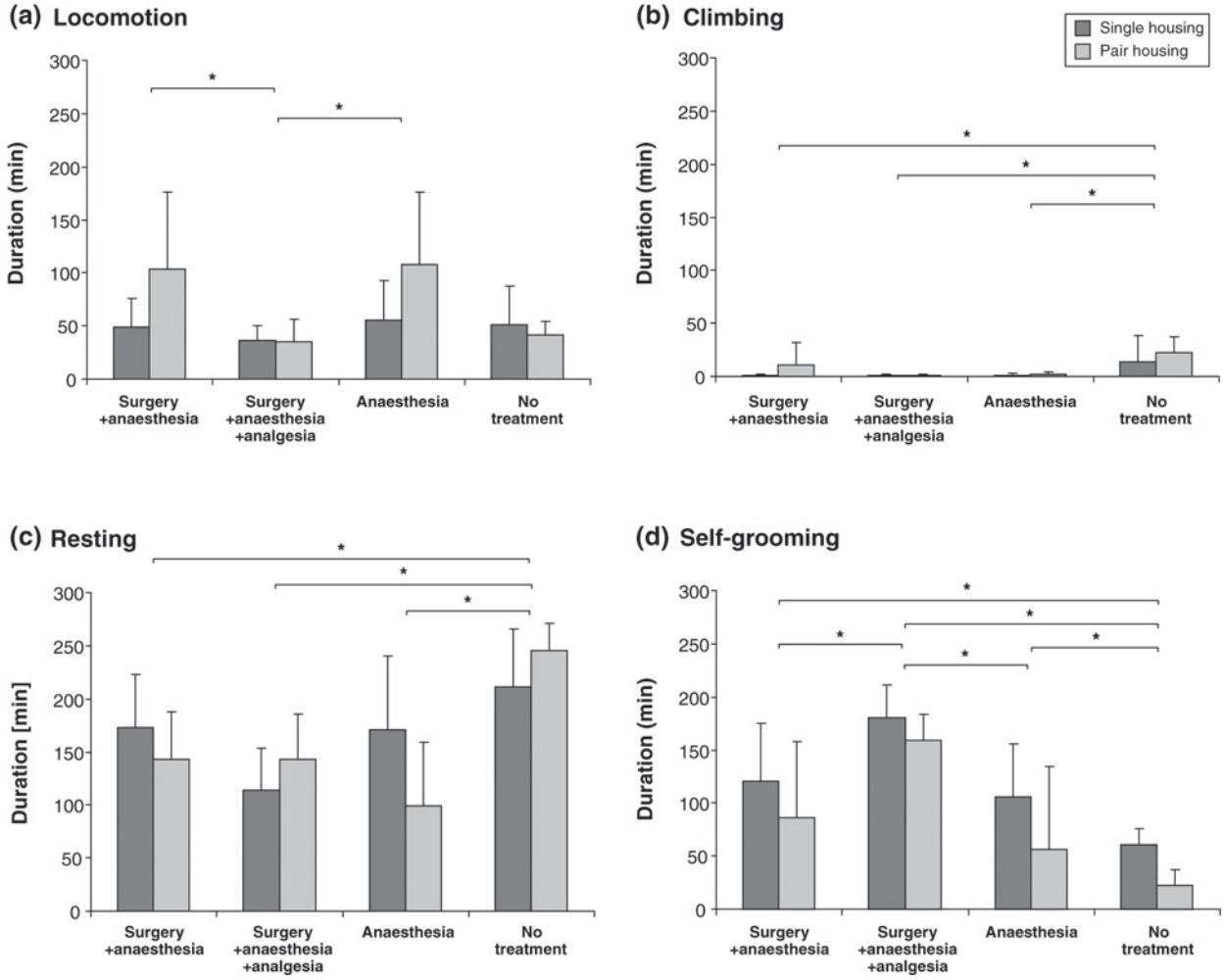

Figure 4 Mean (±SD) duration of individual home cage behaviours mostly responsible for experimental group separation. (a) Locomotion: significant differences in locomotion duration between surgery + anaesthesia + analgesia and surgery + anaesthesia as well as between surgery + anaesthesia + analgesia and anaesthesia groups ($P = 0.042$, $P = 0.015$) were found. Additionally, significant housing effects could be found ($P = 0.025$). (b) Climbing: significant differences between no-treatment animals and experimentally-treated animals were found in climbing (surgery + anaesthesia: $P = 0.041$; surgery + anaesthesia + analgesia: $P = 0.001$; anaesthesia: $P = 0.002$). (c) Resting: significant differences between no-treatment animals and experimentally-treated animals were found (surgery + anaesthesia: $P = 0.001$; surgery + anaesthesia + analgesia: $P < 0.001$; anaesthesia: $P < 0.001$), and a significant interaction between housing and experimental condition ($P = 0.010$). (d) Self-grooming: significant differences between no-treatment animals and experimentally-treated animals were found in self-grooming (surgery + anaesthesia: $P = 0.003$; surgery + anaesthesia + analgesia: $P < 0.001$; anaesthesia: $P < 0.001$). Additionally, significant differences in self-grooming duration between surgery + anaesthesia + analgesia and surgery + anaesthesia as well as anaesthesia groups ($P = 0.002$, $P = 0.011$) were found. Significant results are marked with * ($P \leq 0.05$)

were only anaesthetized or received pain treatment after surgery. Thus, the increase in activity was presumably caused mainly by anaesthesia, while mice in pain seem to spend slightly more time in inactivity.

These observations were in accordance with discriminant analysis, which showed a significant contribution of the analysed home cage behaviours to group separation. Behaviours contributing most to this separation were locomotion, resting, climbing, self-grooming and, to a lesser degree, drinking and eating. Less resting and climbing were correlated with surgery and anaesthesia. Climbing duration was shorter in all groups compared with non-treated mice. This indicates that reduced climbing activity in our study was not due to abdominal pain[12] but rather to general impairment after anaesthesia. Eating, drinking and self-grooming were more prevalent in experimental groups that underwent anaesthesia or surgery. Changes in these behaviours may be consequences of the anaesthetic and surgical procedures. The prolonged eating and drinking duration in some conditions, especially in single-housed mice, might indicate increased food and water consumption that may help to reconstitute the animals' health after an exhausting event. Eating and drinking increased mainly in animals that were only anaesthetized or received pain treatment after surgery, whereas animals without pain treatment did not increase their food and water intake compared with non-treated mice. This low food and water intake is probably a sign of postsurgical pain in these animals. As self-grooming was not increased after surgery without pain treatment compared with the other treated groups it is unlikely to be a specific sign of postsurgical pain. Therefore it could be correlated with the animals' general wellbeing after anaesthesia as well as increased attention to the shaved operation field,[21] the wound or the eye ointment used.

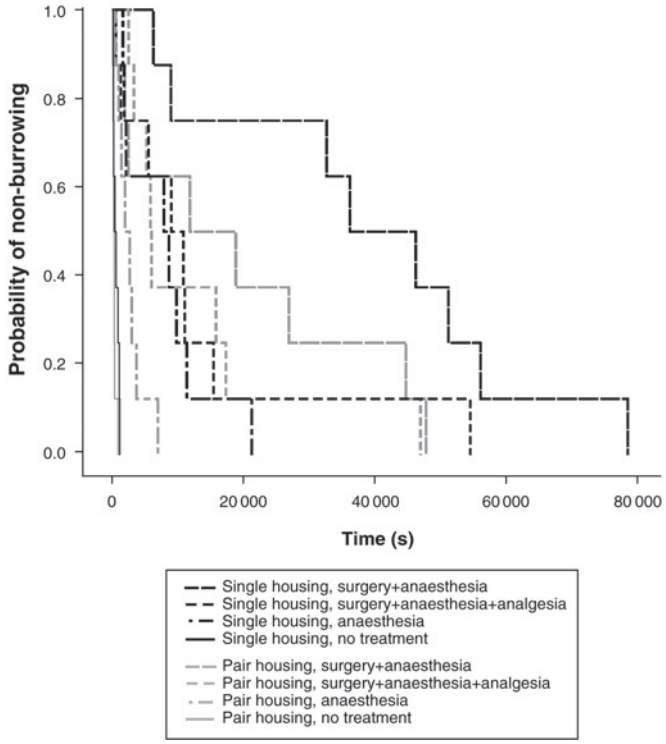

Figure 5 Kaplan–Meier analysis of latency to burrow. In both housing conditions, no-treatment animals had significantly shorter latencies compared with all other groups ($P < 0.001$). Analyses showed significant differences for pairs when surgery + anaesthesia and surgery + anaesthesia + analgesia groups were compared with anaesthesia group ($P = 0.03$, $P = 0.014$), but a significant difference only in single-housed mice when comparing surgery + anaesthesia and anaesthesia groups ($P = 0.003$). Latencies were mainly shorter for pairs compared with single-housed mice, which was significant after surgery + anaesthesia and anaesthesia ($P = 0.021$, $P < 0.001$)

Nest building behaviour has been identified in several publications as a useful estimate of wellbeing and pain.[12,19,22] Even though discriminant analysis did not reveal it as an important behaviour, we observed a decrease in this behaviour after surgery and anaesthesia. A slight gradation in nest building behaviour was seen in pair-housed mice, with longer durations in animals that received pain treatment after surgery and longest durations in anaesthesia-only and non-treated animals.

To summarize, even though socially- and individually-housed mice did indeed differ in several behaviours, none of these differences were significantly relevant in the experimentally-treated groups.

Burrowing behaviour is a highly motivated behaviour that has been shown to decrease after painful surgical interventions.[15] In our study, burrowing performance ranged from short latencies of non-treated animals to an intermediate level following anaesthesia only, to a pronounced prolongation of latency to burrow after surgery without pain treatment. These findings, in accordance with previous studies,[15] indicate an anaesthesia effect as well as the occurrence of pain in animals after surgery. While in both housing conditions non-treated animals had similar short latencies, following treatments latencies to burrow increased more strongly in single animals compared with pairs. The shorter latencies in treated pairs compared with treated single-housed animals hint at a better performance of pairs in the burrowing test, although both cage mates experienced the experimental procedures. This decreased interest in burrowing activity is likely correlated with better coping in pairs after experiments.

Interestingly, after experiments, pairs showed a dramatic decline in social interactions, with only short and rare bouts of social behaviours. Healthy mice show a strong preference for sleeping together in one place;[23] indeed, social resting was the social behaviour most commonly observed in non-treated mice in our study. The strong decrease in social behaviour was due mainly to an increase in self-grooming and individual resting. However, long-term observation revealed social resting even after major surgery.[12] As expected, pair-housed females displayed only very few socio-negative behaviours; hence, the risk of social stress or injury seems to be negligible in female mice after surgery.

As social behaviours almost disappeared, no actual signs of positive social interaction could be found that could hint at observable social support in the postsurgical phase. But while the proximate effects of housing conditions may not be clearly evident, they may affect how animals respond to surgical stressors in the long term. This is in line with our analysis of burrowing performance and is supported by longer term studies showing that a social partner can act as a support after abdominal surgery[11] or telemetry transmitter implantation.[12] Our behavioural observations are limited by a short observation period of only 6 h. We assume that postsurgical pain and impairment of general condition following minor surgery lasts longer than our observation period, possibly up to 24 h.[19] Therefore, we cannot exclude that any recovery-supporting effects of social housing may have been more obvious in the longer term.

It is important to consider that our animals were housed in harmonious groups before and after the experimental period and were always in acoustic and olfactory contact with other mice. Moreover, we used only female mice, which inevitably hamper the transfer of our results to male mice because group-housed males tend to fight, especially when re-grouped after transient individual housing. However, male mice also show a preference for social contact, preferring to sleep in proximity to a familiar male[23] and seem to profit from social housing after challenging events like stroke or nerve injury.[24,25]

In conclusion, although behaviours related to subjective wellbeing of mice decreased after surgery and anaesthesia, most behaviours, except burrowing performance, were too variable to reveal significant differences between housing conditions. From the overall results of our study, we cannot claim that postsurgical social housing is truly superior over single housing. Nevertheless, the observed changes in burrowing performance suggest a tendency towards better wellbeing in pair-housed animals after surgery. Thus, although both housing conditions are acceptable in female mice at least for 6 h after surgery, social housing might be in general preferable. Conversely, single housing has the advantage of allowing accurate monitoring of individuals,[26] which is advised in many situations, e.g. humane endpoint anticipation. Therefore, in our view, the

decision on how to house female mice after surgery should be made on a case-by-case basis, considering various aspects of laboratory routine, legislation and – of course most importantly – possible impacts on animal wellbeing.

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Chapter 6: Housing of female mice in a new environment and its influence on post-surgical behaviour and recovery

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Housing of female mice in a new environment and its influence on post-surgical behaviour and recovery

Key words: Mice; housing conditions; well-being; refinement; behaviour; burrowing

Abstract

The transportation of mice into a new clean cage after surgery is a standard procedure but might have detrimental effects during the critical post-surgical recovery phase. To analyse the effect of post-surgical housing, female C57BL/6J mice housed in their familiar home cage or in a new environment after minor surgery +/- analgesia, anaesthesia only or no treatment were monitored using non-invasive methods during the immediate postsurgical period to assess pain and general impairment. Behavioural investigations and burrowing test revealed no significant differences between housing conditions in untreated mice. While no appearance or posture abnormalities were observed post-experiment, home cage behaviors were affected distinctly. Behavioural rhythmicity was disrupted, and behaviours related to well-being, such as burrowing performance, were less compared to untreated mice. Burrowing latency ranged from an intermediate level following anaesthesia only and surgery with analgesia, to pronounced prolongation after surgery without analgesia in animals housed in their home cage, while after all experimental treatments burrowing latency in animals in new cages was prolonged dramatically. General activity and climbing behaviour in treatment groups housed in new cages tend to be higher compared to animals in familiar cages, leading to significant interactions between housing and treatment conditions ($p = 0.006$; $p = 0.014$). These behavioural differences in animals housed in a new environment compared to animals housed in their familiar environment might be interpreted as signs of reduced well-being, agitation and restlessness in the new cages and may hint that animals cope better with surgical stress when housed in their familiar environment. The post-surgical transport to a new and clean cage might therefore be an additional stressor after an exhausting event and may affect recovery.

Introduction

Laboratory mice are housed under standardized husbandry conditions. In this environment, olfaction probably remains the most significant sense for the animal. Scent marks, originating from urine smears or other glandular sources of secretion such as salivary, plantar or preputial glands and deposited on the substrate, represent a major source of information (1, 2). Many aspects of mouse behaviour rely on their ability to use odour cues, for example to distinguish among individuals, which is essential for maintenance of stable groups, recognition of offspring or mates, advertisement of dominance over a territory as well as for reproduction (3-5). Olfactory cues are also used for orientation and to enhance the detection of novel objects (6).

Two common and rather drastic disturbances of these cues that nearly all mice in the laboratory undergo are cage cleaning and in-house transportation. Cage cleaning normally includes the change of the cage, the removal of all its contents and the transport of the mice into a new cage with fresh bedding and other fresh or autoclaved material. While this procedure is essential for hygiene, it disrupts the olfactory cues of mice and has often been described as a repetitive and frequent stressful event in the lives of laboratory rodents (1, 3, 7). It is known that long-term frequent cleaning of cages causes chronic stress and depresses body weight gain in mice (8). In-house transport to an experimental laboratory or another animal room results in significant increase in plasma

corticosterone concentration in mice and a decrease in thymus gland weight, leukocyte and lymphocyte count, and was therefore considered to be a stressful stimulus in mice (9).

The transportation of an animal after surgery into a new clean cage is a standard procedure in many facilities for several reasons, e.g. the potential health risk of soiled bedding. This procedure combines both stresses of in-house transport and cage cleaning and probably has a comparable or even higher impact on the animal. This procedure may therefore have detrimental effects on the animal during the critical post-surgical recovery phase.

Although the proximate effects of housing conditions on the animal's internal state may not always be obvious, they might affect the way animals respond to additional stressors. For example, Tuli and co-workers have shown that animals in new cages were more sensitive to transportation stress, with mice housed in their home cage recovering faster from this stressor (10). These results led to the suggestion that housing in a new cage may hamper the animal's ability to cope with, and increase the vulnerability to, additional stressful episodes. Surgery and the post-surgical recovery phase represent stressful episodes for mice. Hence, housing conditions may influence an animal's vulnerability to surgical stress and may interfere with post-surgical recovery.

Here, we aimed to analyse the potentially beneficial effect on recovery of post-surgical housing in the home cage by comparing female C57BL/6J mice housed in their familiar home cage or in a new environment after minor surgery. To assess the impact of surgery and different housing conditions on well-being, we used a range of non-invasive behavioural measurements that can be applied in the animals' cage without provoking additional stress. Burrowing performance, changes in home cage behaviours and classical indices like clinical symptoms, overall appearance and body weight should allow recognition not only of post-surgical pain but also impairment of general condition, thus providing a broad picture of the animal's recovery.

We hypothesise that signs of pain and impaired well-being should be reduced in mice housed in their home cage if housing conditions with a stable physical and olfactory environment are beneficial to post-surgical recovery.

Animals

Ethics statement

The animal housing and experimental protocols were approved by the Cantonal Veterinary Department, Zurich, Switzerland, under license no. ZH 120/2008, and were in accordance with Swiss Animal Protection Law. Housing and experimental procedures also conform to European Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 2011).

Animals & Housing

The animals were 64 female C57BL/6J obtained from our in-house breeding facility at the age of 6–8 weeks.

Animals' health status was monitored by a health surveillance program according to FELASA guidelines throughout the experiments. The mice were free of all viral, bacterial, and parasitic pathogens listed in FELASA recommendations, except for Helicobacter species (11).

All animals were housed in groups of three to six animals for at least 3 weeks prior to testing in our animal room. Animals were kept in Eurotype III clear-transparent plastic cages (425 mm × 266 mm × 155 mm) with autoclaved dust-free sawdust bedding and one nestlet™ (5 cm × 5 cm), consisting of cotton fibres (Indulab AG, Gams, Switzerland) as nest building material. They were fed a pelleted and extruded mouse diet (Kliba No. 3436, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to sterilized drinking water. The

light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 Lux in the cage). The temperature was 21 ± 1°C, with a relative humidity of 55 ± 10%, and with 15 complete changes of filtered air per hour (HEPA H 14 filter). The animal room was insulated to prevent electronic and other noise. Disturbances, e.g. visitors or unrelated experimental procedures in the animal room, were not allowed.

Materials & Methods

Experiments

Experimental housing and setup

During the whole experimental period animals were housed under standardized conditions as described above with the burrowing test setup in addition. As burrowing apparatus, a plastic bottle (standard opaque water bottle, 250 ml, 150 mm length, 55 mm diameter) filled with 138–142 g of food pellets identical to those of the animal's normal diet was used. An additional empty bottle of the same dimensions was provided to serve as a shelter (for detailed information, see (12)).

For acclimatization, animals were housed individually for 3 days under these conditions before experiments started. The animals had no prior experience with behavioural testing.

Experimental design

Mice were observed directly after the experimental procedure. 32 mice were housed in their familiar home cage during the observation while the other 32 mice were transported directly after the experimental procedure to a new clean cage containing a similar, but clean, set up as during acclimatization. Eight mice of each housing condition were allocated randomly to one of three experimental groups: (1) surgery + anaesthesia (mice underwent anaesthesia and surgery without analgesic treatment); (2) surgery + anaesthesia + analgesia (mice underwent anaesthesia and surgery with analgesic treatment); (3) anaesthesia only; or received no experimental treatment.

Experiments and data acquisition

The experiment began with a subcutaneous injection of 2 µl/g body weight of phosphate buffered saline (PBS) for the surgery + anaesthesia and anaesthesia only groups. In the surgery + anaesthesia + analgesia group, 5 mg/kg body weight of the analgesic carprofen (Rimadyl™, Pfizer Inc., New York, NY, USA) was diluted in PBS and injected as 2 µl/g body weight. The animals were transferred 45 minutes later in transport cages to the nearby operating theatre. Mice were anesthetized with sevoflurane (Sevorane™, Abbott, Baar, Switzerland) as mono-anaesthesia. The anaesthetic gas was provided with a rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland); oxygen was used as carrier gas. After induction of anaesthesia in a Perspex induction chamber (8% sevoflurane, 600 mL/min gas flow), animals were transferred to a warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at 39°± 1°C to ensure constant body temperature, and anaesthesia was maintained via a nose mask (4.9% sevoflurane, 600 mL/min gas flow). Eye ointment was applied, the fur was clipped and the operating field disinfected with ethanol (70%) in all animals. Mice in both surgery groups underwent a one-side sham embryo transfer. The incision in the abdominal muscle wall was closed with absorbable sutures (Vicryl™, 6/0 polyglactin 910, Ethicon Ltd, Norderstedt, Germany), and the skin was closed using skin staples (Precise™, 3M Health Care, St Paul, MN, USA). Surgery was completed within 6–8 min in both surgery groups. Anaesthesia lasted 14–16 min in all groups. Animals were allowed to recover for 15–20 min on the warming mat before being transferred back to the animal room for subsequent behavioural observation.

Experimental treatments were completed at the start of the light phase by returning each mouse from its transport cage to the observation cage. This was the animal's familiar home cage containing the refilled burrowing test apparatus or a new clean cage containing a new and filled burrowing test apparatus. In the case of non-treated mice in their familiar home cage the test apparatus was just refilled. Observation began by starting the digital video recording.

Behavioural analysis

Home cage behaviours

The recorded video sequences were analysed continuously using ObserverXT™ software (Noldus, Wageningen, Netherlands) for the first 6 hours of the light phase. Durations of behaviours (resting, locomotion, self grooming, eating, drinking, climbing, burrowing, nest building; Table 1), and numbers of resting bouts were measured. General activity was calculated by summarizing all active behaviours (i.e. all home cage behaviours except resting). Non-defined behaviours were not recorded.

Table 1*: Ethogram of home cage behaviours according to Van Oortmerssen (1970).*

home cage behaviours	
resting	sitting or lying flat or curled up, sometimes with the eyes closed or nearly closed (includes sleeping)
locomotion	walking, running, jumping
self grooming	bouts of wiping, licking and nibbling the own fur with forepaws and tongue
eating	series of movements resulting in ingesting food
drinking	taking in liquids with series of licking movements of the tongue
climbing	climbing with all four feet at the cage grid
burrowing	all behaviours linked with emptying the burrowing apparatus (digging, carrying etc. of material)
nest building	all behaviours linked with nest building (arranging, pulling in, fraying etc.)

Burrowing performance

The burrowing test determines burrowing performance and can be used as simple method to assess post-surgical impairment in mice. Good performance in this test is defined as short latency to remove items from a tube-like apparatus (burrowing) (12). Burrowing was defined as the removal of more than three pellets from the apparatus within 10 seconds. The latency to burrow of each animal was measured. Measurement of latency was continued for 24 hours if the animals did not start to burrow within the six hours of behavioural analysis.

Clinical investigation

Animals were weighed at the beginning of the light phase 24 hours before, and 24 and 48 hours after experiment and observed for 20-30 seconds before, during and after weighing. According to a routinely used scoring system documenting the general condition of an animal (13), abnormalities of body condition (e.g. sunken flanks), fur condition (e.g. ruffled coat), eyes (e.g. discharge), breathing (e.g. irregular) and posture (e.g. hunched back) were registered, and wound healing, spontaneous behaviour and movement were assessed.

Statistical analysis

Statistical analyses were performed with SPSS 20.0 software (IBM, Armonk, NY, USA).

All data was tested for normal distribution and homogeneity of variance (Shapiro-Wilks, Levene's test). If necessary, data was log (X+1) transformed to meet assumptions of statistical tests.

Mean and standard deviation (SD) of durations of home cage behaviours and numbers of resting bouts were calculated.

Discriminant analysis was used to determine behaviours mainly responsible for group separation. The determined behaviours were further analysed using multivariate general linear model (GLM) with experimental group and housing as fixed factors. Post hoc tests (Bonferroni) were used for comparisons between experimental groups.

Mean duration of resting bouts was calculated by dividing resting duration by number of resting bouts. Activity duration and mean duration of resting bouts were compared between groups using a multivariate general linear model (GLM) with experimental group and housing as fixed factors. Post hoc tests (Bonferroni) were used for comparisons between experimental groups.

Mean and standard deviation (SD) of latency to burrow were calculated. Kaplan–Meier survival analysis was performed to examine the distribution of time to effect (latency to burrow). To test whether latency to burrow differed statistically between experimental groups or housing conditions, a log rank significance test was performed.

Significance for all statistical tests was established at $p \leq 0.05$.

Results

Influences of housing conditions on healthy mice

Behavioural differences between healthy, i.e. non-treated, mice were minor and none of the behaviours analysed showed a significant housing effect (see Figure 1, Figure 2, Table 2 and Table 3).

In both housing conditions, animals showed a short burrowing latency in no treatment groups (familiar cage 8 +/- 6 min; new cage 6 +/- 6 min, Figure 3).

	familiar cage				new cage			
	surgery + anaesthesia	surgery + anaesthesia + analgesia	anaesthesia	no treatment	surgery + anaesthesia	surgery + anaesthesia + analgesia	anaesthesia	no treatment
	behaviours (mean +/- SD)							
resting [min]	173 +/- 50	114 +/- 40	178 +/- 79	198 +/- 66	155 +/- 47	125 +/- 31	100 +/- 44	247 +/- 38
locomotion [min]	48 +/- 27	33 +/- 15	56 +/- 38	57 +/- 23	41 +/- 21	46 +/- 17	34 +/- 30	49 +/- 19
self grooming [min]	105 +/- 61	180 +/- 31	174 +/- 29	49 +/- 27	135 +/- 51	144 +/- 41	135 +/- 24	25 +/- 19
eating [min]	9 +/- 10	25 +/- 17	19 +/- 18	16 +/- 12	9 +/- 8	18 +/- 5	24 +/- 13	8 +/- 6
drinking [min]	1 +/- 1	3 +/- 3	2 +/- 1	1 +/- 1	1 +/- 1	3 +/- 1	2 +/- 1	1 +/- 1
climbing [min]	1 +/- 1	1 +/- 1	1 +/- 2	18 +/- 22	3 +/- 5	7 +/- 11	32 +/- 43	8 +/- 7
burrowing [min]	2 +/- 4	1 +/- 2	3 +/- 3	8 +/- 3	2 +/- 3	2 +/- 3	2 +/- 4	6 +/- 2
nest building [min]	4 +/- 6	1 +/- 1	3 +/- 5	10 +/- 9	8 +/- 10	6 +/- 10	17 +/- 18	11 +/- 8

***Table 2**: Mean duration +/- SD of home cage behaviours in minutes for animal housed in their familiar home cage or in a new cage after experimental treatment or no treatment.*

Influences of experimental treatment on mice

After experimental treatment animals showed no abnormalities in appearance, posture or spontaneous movements. No complications in wound healing after surgery were observed. No significant changes in body weight compared with one day prior to experimental treatments were seen at either one or two days after treatment. Clinical investigation revealed unaltered general condition scores in all groups.

Mean durations of the observed behaviours of treated and non-treated mice in both housing conditions are shown in Table 2.

Discriminant analyses were performed with these behaviours for animals housed in their familiar home cage or a new clean cage revealing that several behaviours contributed to the significant separation of experimental groups (familiar cage: Wilks' lambda, function 1, $p = 0.001$; new cage: Wilks' lambda, function1, $p < 0.001$, function 2 = 0.017). GLM was then performed with the main behaviours found to be contributing to experimental group separation in discriminant analyses (duration of climbing, eating, burrowing, self grooming; Figure 1) and additionally with general activity and mean resting bout duration (Figure 2) to test for significant differences between treatments and housing conditions.

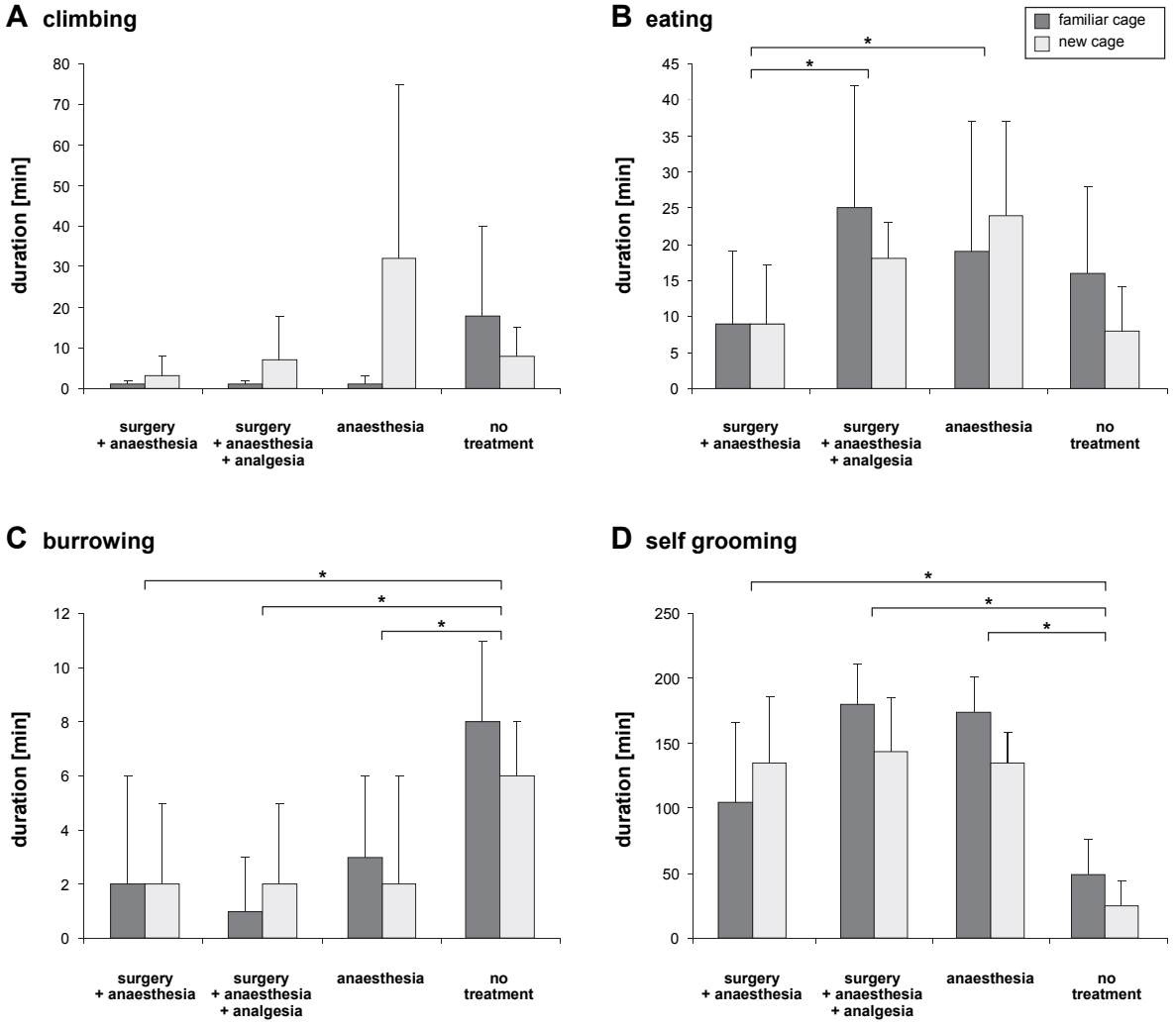

Figure 1: A Climbing: *A significant interaction between treatment and housing was found ($p = 0.014$); therefore no post hoc test was conducted for this behaviour.* ***B Eating:*** *Eating duration showed significant differences between surgery with and without analgesia ($p = 0.028$) and surgery without analgesia and anaesthesia only ($p = 0.022$);* ***C Burrowing:*** *Burrowing duration was significantly shorter in treatment groups (no treatment vs. surgery $p < 0.001$, no treatment vs. surgery + analgesia $p < 0.001$, and no treatment vs. anaesthesia $p = 0.001$);* ***D Self grooming:*** *Grooming duration was significantly higher in treatment groups (no treatment vs. surgery $p = 0.009$, no treatment vs. surgery + analgesia $p < 0.001$, no treatment vs. anaesthesia $p < 0.001$).*

Main effects and interactions of the factors housing and treatment

No main effect of the factor housing could be shown in any of the analysed behaviours, while the factor treatment had a significant effect on durations of all behaviours with the exception of climbing duration (Table 3).

Significant interactions between the two main factors housing and treatment were found in climbing ($p = 0.014$) and activity ($p = 0.006$) durations (Table 3, Figure 1 A and Figure 2 A). Because of the significant interaction post hoc test were not performed for these behaviours but the following tendencies could be observed: While climbing duration was shorter in treated animals housed in familiar cages compared to non-treated mice, the differences were less pronounced in animals in new cages. Climbing durations of animals that underwent anaesthesia only were even higher in this housing condition compared to non-treated animals. General activity was higher following treatments compared to non–treated animals. In the new cages this difference tended to be higher than in the familiar cages.

Table 3: *The effects of housing and treatment on analysed behaviours. When interactions were significant, main effects were not reported because they are abundant.*

behaviour (duration in min.)	**main effects housing**	**main effects treatment**	**interaction housing*treatment**
climbing	--	--	$F = 3.859$; $p = 0.014$
eating	$F = 0.829$; $p = 0.366$	$F = 4.856$; $p = 0.004$	$F = 0.958$; $p = 0.419$
burrowing	$F = 0.363$; $p = 0.549$	$F = 10.946$; $p < 0.001$	$F = 0.716$; $p = 0.547$
self grooming	$F = 0.899$; $p = 0.347$	$F = 10.877$; $p < 0.001$	$F = 0.789$; $p = 0.505$
activity	--	--	$F = 4.521$; $p = 0.006$
resting bouts	$F = 0.030$; $p = 0.864$	$F = 21.375$; $p < 0.001$	$F = 0.626$; $p = 0.601$

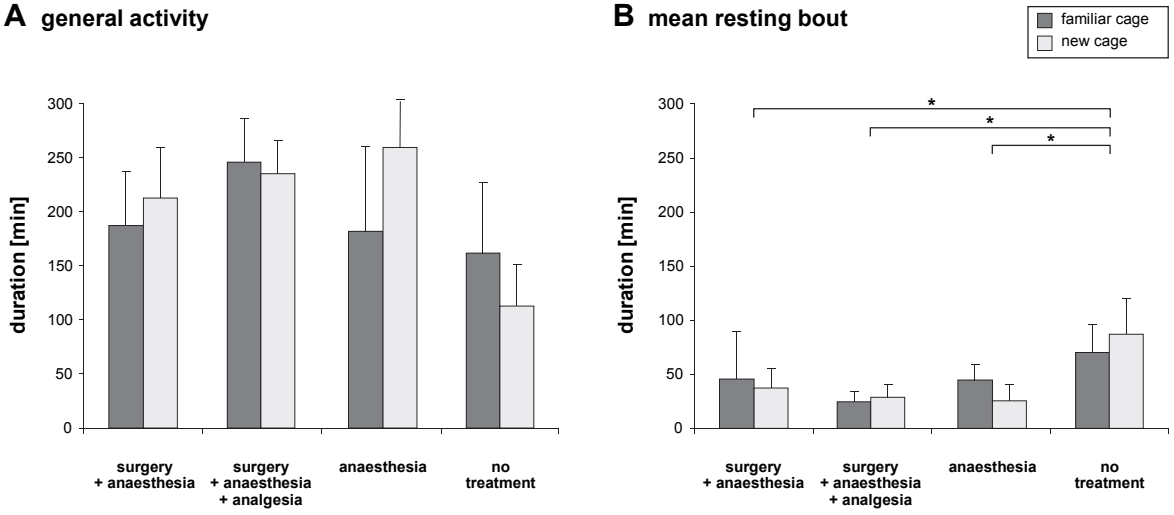

Figure 2: A General activity: *A significant interaction between treatment and housing was found ($p = 0.006$); therefore no post hoc test was conducted for this behaviour.* ***B Mean duration of resting bouts:*** *Experimental treatment groups had significantly shorter mean resting bout durations (no treatment vs. surgery $p < 0.001$, no treatment vs. surgery + analgesia $p < 0.001$, no treatment vs. anaesthesia $p < 0.001$).*

Effects of specific experimental treatments

Eating durations showed a non-significant tendency towards longer durations in the anaesthesia only and surgery with analgesia groups compared to non-treated animals (n.s., $p = 0.124$; $p = 0.156$), while surgery without pain treatment resulted in durations lower or comparable to non-treated animals (n.s., $p = 1.00$). This resulted in significant differences between surgery with and without analgesia ($p = 0.028$, Figure 1 B) and surgery without analgesia and anaesthesia only ($p = 0.022$, Figure 1 B).

Duration of burrowing was significantly shorter in treatment groups compared to non-treated animals (no treatment vs. surgery $p < 0.001$, no treatment vs. surgery + analgesia $p < 0.001$, and no treatment vs. anaesthesia $p = 0.001$, Figure 1 C).

Grooming behaviour was performed for significantly longer times in treatment groups compared to non-treated animals (no treatment vs. surgery $p = 0.009$, no treatment vs. surgery + analgesia $p < 0.001$, no treatment vs. anaesthesia $p < 0.001$, Figure 1 D).

In experimentally treated animals the mean duration of resting bouts in experimentally treated animals was shorter (no treatment vs. surgery $p < 0.001$; no treatment vs. surgery + analgesia $p < 0.001$; no treatment vs. anaesthesia $p < 0.001$, Figure 2 B).

Influences of housing and treatment on burrowing performance

Experimental treatments resulted in prolonged latencies in the burrowing test (Figure 3 A and B). Log rank test following Kaplan-Meier analyses showed significant differences between non-treated animals and the experimentally treated groups under both housing conditions ($p = 0.001$). Animals housed in their familiar home cage showed a pronounced gradation of burrowing latency between treatments. The mean latency of animals that underwent surgery without pain relief was distinctly higher (677 +/- 402 min) than latencies in animals that received analgesia after surgery (310 +/- 340 min) or anaesthesia only (315 +/- 246 min). Animals housed in a new cage after treatment showed similar latencies in both surgery groups (surgery 570 +/- 267 min; surgery + analgesia 531 +/- 411 min) and the highest latency in animals that underwent anaesthesia only (751 +/- 538 min). Log rank test showed that the difference between surgery without analgesia and anaesthesia only groups was significant in animals housed in their home cage ($p = 0.020$, Figure 3 A). Comparing both housing conditions, burrowing latency after anaesthesia only was shorter for mice housed in their familiar home cage compared with mice housed in a new cage ($p = 0.049$, Figure 3).

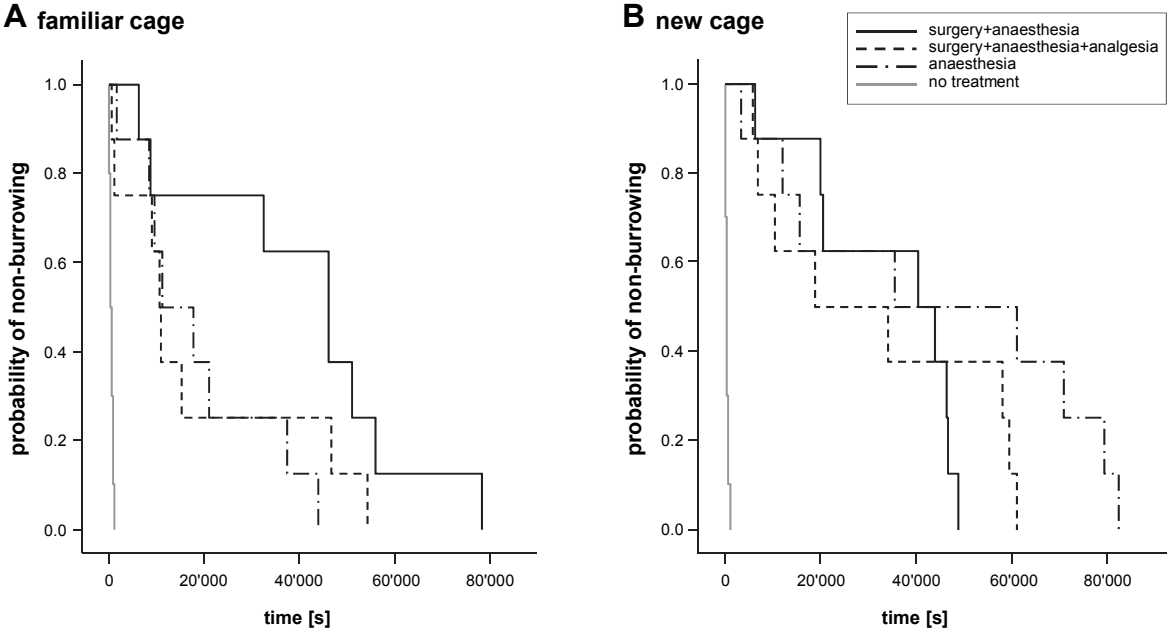

Figure 3: Kaplan–Meier analysis of latency to burrow. A Familiar home cage; B New clean cage. *Significant differences were found between non-treated animals and the experimentally treated groups under both housing conditions ($p = 0.001$). The difference between surgery without analgesia and anaesthesia only groups was significant in animals housed in their home cage ($p = 0.020$). Comparing both housing conditions, latency after anaesthesia only was shorter for mice housed in their familiar home cage compared with mice housed in an new cage ($p = 0.049$).*

Discussion

This study was set up to determine whether postsurgical housing in the familiar home cage is more beneficial for the recovery and well-being of female mice than housing the animals in a new and clean cage after surgery. For this purpose, animals in both housing conditions were monitored closely during the period immediately after surgery or anaesthesia. Behavioural investigations revealed significant differences in most behaviours in experimentally treated groups (surgery with or without analgesia, or anaesthesia only) compared to non-treated mice, while in contrast behaviours showed no significant differences when comparing housing conditions. Nevertheless, significant interactions between housing and treatment in climbing and activity durations as well as differences in burrowing performance occurred that may hint that animals cope better with surgical stress when housed in their familiar environment.

Clinical investigations, focusing on changes in appearance, posture and body weight, carried out daily are standard monitoring tools after surgery. Since no abnormalities were detected with these investigations, we suggest that our model has only a low impact on condition, health and well-being, particularly in comparison with other models of surgery (e.g. (14)).

Behavioural differences between untreated animals under both housing conditions were minor, and none of the analysed behaviours showed a significant housing effect in the statistical analyses.

In contrast, experimental treatments resulted in significant changes in nearly all analysed behaviours compared to non-treated animals under both housing conditions. These distinct changes were expected as we compared healthy animals that were not treated or manipulated at all with animals that underwent at least transport to the nearby operation theatre and inhalation anaesthesia. We assume that these differences can be explained only partly by restraint procedures and manipulations, as standard restraint and injection procedures have been shown to have only short-term impact on mice (15, 16). Studies from our group instead hint that the behavioural effects are due mainly to the impact of anaesthesia (12, 17-19).

While healthy mice mostly rest during the light phase and show a stable circadian rhythm with long resting bouts; disruption of this rhythm might indicate impaired well-being (20). In our study, compared to non-treated animals, overall activity was increased, accompanied by significantly more and shorter resting bouts, resulting in a disruption of the activity rhythm in all treated groups, indicating a decrease in animal well-being due to the treatments.

Discriminant analysis showed a significant contribution of the observed home cage behaviours to group separation. The behaviours contributing most to this separation were climbing, eating, self grooming and burrowing. While there was no main effect of treatment on climbing duration, eating duration was affected. Even though eating behaviour is not necessarily identical to food intake, the non-significant tendency to prolonged eating duration compared to non-treated animals in some conditions might indicate that animals increased their food consumption. This may help to reconstitute the animals' health after an exhausting event. Eating increased mainly in animals that were only anaesthetized or received pain treatment after surgery. This resulted in significant differences to animals without pain treatment that did not increase eating duration compared with non-treated mice. This might correlate with a low food intake and is probably a sign of postsurgical pain in these animals. As self-grooming was significantly more prevalent in all treatments compared to untreated animals, it is unlikely to be a specific sign of postsurgical pain. Therefore it could be correlated with the animals' general well-being after anaesthesia as well as increased attention to the shaved operation field (21), the wound or the eye ointment used.

Burrowing behaviour is a highly motivated behaviour that has been shown to decrease after painful surgical interventions (12, 19). Burrowing duration compared to non-treated animals was significantly shorter and burrowing latency in the burrowing test was significantly longer in all treatment groups in both housing conditions. In animals housed in their familiar home cage, burrowing performance ranged from short latencies of non-treated animals to an intermediate level following anaesthesia only and surgery with analgesia, to a pronounced prolongation of latency to burrow after surgery without pain relief. In accordance with previous studies (12, 19), these findings indicate an anaesthesia effect as well as the occurrence of pain in animals after surgery. While non-treated animals had a similar good burrowing performance with short latencies in both housing conditions, latencies to burrow were dramatically but not in all cases significantly prolonged in animals transferred to a new cage after treatment. In addition to the prolongation of latencies, the transportation of animals to a new cage resulted in latencies to burrow that did not show a clear gradation of the different treatment groups as seen in animals housed in their familiar environment.

Interactions of housing condition and experimental treatment were also seen in other home cage behaviours. The analyses of general activity and climbing behaviour showed significant interactions between housing and treatment. In new cages, activity was higher after treatment compared to non-treated mice, while this difference was distinctly smaller in familiar cages. Climbing durations were shorter in treated animals in familiar cages compared to non-treated mice, whereas higher or comparable in treatment groups in new cages. Because of the significant interactions these treatment differences were not tested for significance. It is known that a disturbed circadian rhythm and decreased burrowing performance might indicate impaired well-being (12, 19, 20, 22). We interpret the relative longer durations of exploratory or flight behaviour (i.e. climbing) and general activity in new cages during the resting phase of the animal as a sign of agitation and restlessness. Increased activity during the natural resting phase might be detrimental for post-surgical recovery. The decreased interest in burrowing activity in animals housed in a new environment might be a consequence of preoccupation with behaviours like climbing. Otherwise, the better performance of animals housed in their home cage may also be a sign that animals in a familiar environment cope better with stressful and exhausting events like surgical procedures, anaesthesia and handling procedures.

These results are in line with other studies that show that even slight changes in a laboratory animal's environment might cause novelty stress and can alter its behaviour during an experiment or produce physiological stress responses (23-25). Our results suggest that, even though housing female mice in a new and clean cage might be not a distressful event per se, post-surgical transfer to a new environment might act as an additional stressor after an exhausting experimental procedure and might be a detrimental factor for a fast and sound post-surgical recovery.

Conclusion

No clear signs of reduced well-being could be observed in healthy female mice placed in new and clean cages. Nevertheless, after experimental treatment, behavioural differences in animals housed in a new environment compared to animals housed in their familiar environment can be interpreted as subtle signs of reduced well-being, agitation and restlessness in the new cages. These results may also hint that animals cope better with surgical stress when housed in their familiar environment. The post-surgical transport to a new and clean cage might therefore be an additional stressor after an exhausting event and detrimental for recovery. We conclude that it might be worthwhile to consider the effects of crucial changes, like cage change, in the animal's physical

environment after experimental procedures to minimize distress for the animals as well as to reduce unwanted variation in research findings.

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CONCLUSION

Assessing animal welfare is crucial in animal experimentation. Defining the welfare state of a sentient animal remains challenging because the various physical and mental systems, processes and responses involved are complex and operate in an interconnected way (12, 13), and reliable welfare indicators are scarce. Considering the remaining difficulties in assessing welfare or well-being in laboratory mice, we aimed to develop easy-to-use and non-invasive behavioural indicators of states such as pain, stress and impairment that affect animal welfare adversely in two clinical settings occurring typically in animal experimentation. These indicators may help to adjust pain management and strain reducing measures as well as in assessing the impact of different procedures and phenotypes on animal well-being to determine prospective and retrospective severity grades. Ideally such indicators should be feasible to use in daily routine by care takers and researchers without the need for specialised expertise.

Following invasive procedures, for example, assessment of pain to adjust analgesic treatment is of high priority. A common feature of current behavioural measures of pain is that they are more sensitive than standard clinical signs. Mouse grimace scale, pain behaviours like stretch and, to some extent, certain vocalizations are the few non-invasive parameters that can measure reliably spontaneously occurring mild-to-severe pain in mice today (24, 48, 59, 65-68). Nevertheless, the main field of application of these methods is more in basic pain research than in the clinical situation at the cage site as they require transfer of the animal into an observation box or the use of specialised equipment (48, 59, 65-68).

Additionally, the clinical situation after surgery as well as during progression of a chronic disease are typical examples of a combination of pain, fear, distress and physical constraint experienced by an animal. Measuring solely pain might give an incomplete picture of the animal's situation, as the animal might still suffer from fear and distress because of experimental housing or from the impact on physiology caused by anaesthesia.

In the presented studies we conducted on-site observations as well as video recordings of natural behaviours such as burrowing and nest building performance, and analysed behavioural profiles and the temporal distribution of changes in these profiles in the animals' home cages to detect indicators of pain and distress but also general impairment to acquire a broad picture of the animals' well-being.

Observations in the home cage are advantageous as they mean minimal stress to the animal and reduce unwanted effects like novelty stress, stress-induced analgesia or other changes in physiology and behaviour that may be caused by the unfamiliar environment of an observation box.

The primary model used to reduce well-being experimentally was a standard surgery protocol combining short inhalation anaesthesia with a one-sided sham embryo transfer in females or one-sided sham vasectomy in males. These are common surgical procedures in the generation of genetically modified mice. Even though both procedures are called minor surgery and analgesia might be withheld it can be assumed that stress, inflammation and at least mild-to-moderate pain are induced.

In our studies, performing these procedures resulted in a minimal response of classical indicators of reduced well-being like body weight and clinical signs as well as specific pain behaviours like stretch and press. Such absence of obvious signs of pain after minor and moderate surgery in laboratory mice is commonly known from

anecdotal evidence and from experiences in the laboratory routine of embryo transfer and vasectomy worldwide. Unlike these classical signs of reduced well-being, post-surgical home cage behaviours were affected significantly in all our studies.

Clear changes in resting, locomotion and self-grooming, and in one study also climbing, behaviour were seen; these were on a moderate level after anaesthesia and marked after surgery compared to baseline and lasted for up to 18 hours when observation ended. Although not significant, a tendency towards more marked changes was seen in surgery without pain treatment compared to surgery with pain relief.

While resting and locomotion were decreased as well as increased at several time points contradictory to their circadian rhythm, self-grooming was increased independently of the time of observation. As pain sensitivity as well as the display of pain signs is known to be dependent on circadian rhythm (94-96), our results highlight the necessity for knowledge of species-specific circadian rhythms of behaviours as well as the importance of determining the proper time of day for behavioural assessment. The fact that behavioural effects are not displayed uniformly over 24 hours, and that changes due to pain or impairment might easily be missed, makes the use of behaviours like locomotion and resting in routine assessment difficult, while an increase in self-grooming might be used as a rather unspecific indicator of reduced well-being at all times of day after surgery and anaesthesia procedures. In general, changes in behavioural profiles after anaesthesia and surgery with or without pain relief were notable and this impact of anaesthesia and surgery should be considered when interpretating research data (see also supplementary data on telemetric measures after anaesthesia and surgery).

It is very likely that the overall poor discriminatory power of these very elaborate methods is due to the high inter-individual variability of behaviour duration even in inbred mice, which makes this parameter unsuitable for standard welfare assessment. As durations of behaviours might be an inappropriate tool for reliable and easy welfare assessment, we conducted two home cage based behavioural tests using species-typical and spontaneously occurring behaviours of mice in subsequent studies.

Nest complexity scoring and the burrowing test and are based on two highly motivated behaviours of mice that persist also in the laboratory environment. Complex nests as well as good performance in the burrowing test are assumed to be signs of good well-being in adult mice of both sexes (97).

Assessing the complexity of nests has been proposed by several authors to be a useful method with which to detect pain (54, 93, 97) or neurodegenerative diseases like Parkinson and Alzheimer in murine models (84, 98). Our study aimed to establish nest complexity scoring to assess surgical impact; a scoring scheme as well as the most suitable scoring time point were thereby established. A distinct reduction in nest complexity could be observed after surgery or anaesthesia, correlating with the degree of invasiveness. While anaesthesia alone had a moderate impact on nest construction, only less than 10% of animals had identifiable nests after surgery. This massive decrease in nest complexity could not be alleviated in a significant manner by analgesia even if a greater dosage than that proved effective in other studies was administered (e.g. (54)). Thus, this study lacks proof that pain caused the reduction in nest building performance and suggests that other impacts related to the experimental protocol like handling stress or mechanical impairment might be responsible for changes in nest building behaviour. Additionally, nest building is a highly complex form of interaction with the environment (85, 99) that is dependent on circadian rhythm and sensitive to time of testing. It is likely that a multitude of internal and external factors could have increased variability in our data and thus resulted in a loss of sensitivity towards the experimental treatments. Nevertheless, even though nest complexity scoring might not be sensitive

enough to discriminate significantly between different detrimental impacts, it detects decreased general condition robustly and may provide a very simple short-term retrospective indicator. Impairment of general condition might then be assessed in depth with more specific indicators. European and Swiss legislation define nest building material as a basic requirement for mice. On these grounds, the presence and condition of a nest can be assessed effortlessly in the animals' home cage. Additionally, nest complexity scoring, based on normal behaviour in the home cage does not require special apparatus, means no additional stress to the animals and can be assumed to be robust in different strains, sexes, and settings, which leads to good transferability of the method.

In the burrowing test we analysed a spontaneous behaviour of mice that is, like nest building, provoked by certain materials in the animals' home cage. However, unlike nest material, the possibility to dig burrows is usually not provided to laboratory mice in standard housing systems. In our test setting, where burrowing was allowed by providing appropriate material, changes in burrowing performance proved to be sensitive to the impact of anaesthesia, pain and the analgesic effect of a standard non-steroidal analgesic drug. A distinct gradation in burrowing performance ranging from an intermediate level after anaesthesia only to a distinct prolongation of latency after surgery could be observed in all studies conducted with this test. Moreover, analgesia after surgery was associated with a significantly earlier onset of burrowing compared to surgery without pain relief, thus proving that discrimination between pain and other detrimental impacts of the experimental protocol is possible with this behavioural indicator. The prolongation of latency to burrow might also hint at the duration of post-surgical pain and the progression of recovery from surgery and anaesthesia.

Additionally, burrowing performance successfully detected the onset of disease in our second model of reduced well-being. Inflammatory bowel disease is frequently investigated by murine models of colitis, but sensitive non-invasive measures of animal welfare in these models are rare. In our study the onset of intestinal inflammation correlated with a significant decrease in burrowing behaviour. Thus burrowing performance might be useful to confirm the onset of inflammation in mice as early as the other disease-specific parameters currently used, which are invasive or at least need restraint and manipulation of the animal. The analyses of burrowing performance in murine models of colitis might add a motivational or mental component to standard health parameters like disease-specific indicators or body weight measurements, which have the potential to be affected significantly by the diarrhoea occurring in this model.

Overall, burrowing performance appears to be a robust indicator of well-being after surgery and in several disease models like colitis or pathological disorders of the brain (85, 87, 88, 90, 99, 100). Although this test can be used to detect a variety of constraints, like inflammation, pain, anaesthesia effects, brain lesions etc., it can be used to discriminate between pain and other experimental impacts as shown in our studies. Advantages of the test are that it is an easy to use and relatively low cost procedure. Additionally, it shows high transferability to different mouse strains (data not published, see figure 9) as well as other rodent species (101). Repeated testing of individuals is also possible as it resulted in only slight oscillation of performance level in our studies as well as in the hands of other authors (97, 102) and the testing of group housed animals is also possible. Consequentially, burrowing performance is now used increasingly to test the efficiency of analgesics in different pain models (103-107).

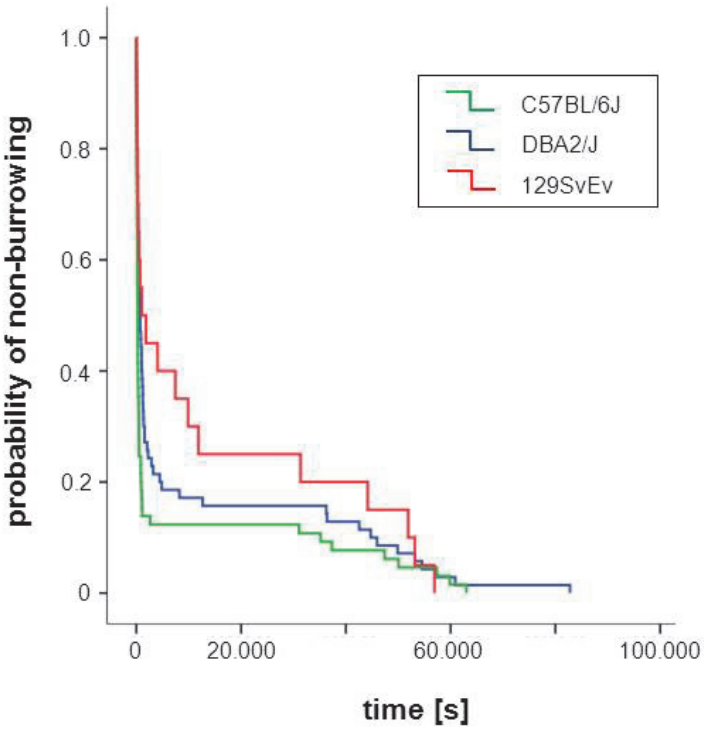

Figure 9: *Kaplan-Meier analysis of burrowing latencies of healthy, individually housed, male mice of the strains C57BL/6J, DBA2/J and 129SvEv. Despite strain differences, the majority of animals of all three strains start burrowing within less than one hour.*

Housing and husbandry have a major impact on a laboratory animal throughout its life (92), and post-procedural recovery supporting measures might be more than just pain relief. Housing conditions therefore may play a major role as an accompanying strain-reducing measure during and after exhausting, stressful or painful experimental procedures. We monitored the immediate post-surgical period to assess pain and general impairment under different housing conditions (social or individual housing, new or familiar cage). While no differences in the behaviour of healthy mice under different housing conditions were detected with our behavioural tools, post-procedure displays differed subtly in most behaviours but significantly in burrowing performance. Despite a tendency towards better recovery in pairs, we found no distinct negative effect of individual housing. In conclusion, individual housing is acceptable during the period immediately following minor surgery, though social housing is always preferable in female mice. Additionally differences in animals housed in a new environment compared to animals housed in their familiar environment might be interpreted as signs of reduced well-being, agitation and restlessness in the new cages and may hint that animals cope better with surgical stress when housed in their familiar environment. The post-surgical transport to a new and clean cage might be an additional stressor after an exhausting, stressful or painful event and may affect recovery.

While most home cage behaviours might only be useful to assess general changes in animal well-being, burrowing performance proved to be a sensitive tool to assess pain, stress and impairment in two clinical settings and under different housing conditions occurring in animal experimentation. Therefore it might be a useful tool with which to adjust pain management and strain-reducing measures as well as to assess the impact of different procedures and phenotypes on animal well-being to determine the grade of severity both prospectively and retrospectively. Burrowing performance, nest complexity and some of the tested home cage behaviours might be assessed as part of the "score sheets" that are typically used in welfare assessment to give a reliable hint as to the

animals' condition during and after experimental interventions. These behaviours might add a motivational aspect to this overview, which is often largely focused on health indicators, leading to assessment schemes that use a wide range of parameters covering the principal constituents of animal welfare and compile a mental and physical profile of the animal (figure 10).

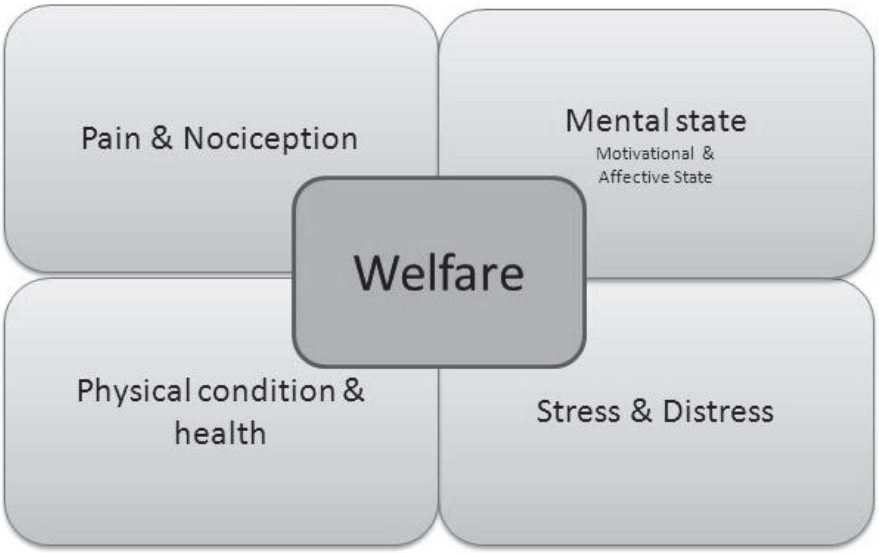

Figure 10: *The welfare state of a sentient animal is very complex, with various interconnecting systems, processes and responses. Animal welfare can be influenced by several conditions like absence or presence of pain or distress and the physical or mental state of the animal as well as the interactions of these conditions.*

The emphasis of welfare research in the past has remained heavily on identifying negative indicators of poor welfare (e.g. aberrant behaviours). It is becoming increasingly apparent that positive welfare indicators, like burrowing performance, offer an approach for developing welfare systems in a more proactive manner. Therefore, future research should be directed to applied animal care, using methods that can be refined not only to minimise pain and suffering but also to provide circumstances and housing conditions that actively promote welfare and help animals to cope better with exhausting, stressful, frightening or painful experimental procedures.

SUPPLEMENTARY PUBLICATIONS

Video Article

Implantation of Radiotelemetry Transmitters Yielding Data on ECG, Heart Rate, Core Body Temperature and Activity in Free-moving Laboratory Mice

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Abstract

The laboratory mouse is the animal species of choice for most biomedical research, in both the academic sphere and the pharmaceutical industry. Mice are a manageable size and relatively easy to house. These factors, together with the availability of a wealth of spontaneous and experimentally induced mutants, make laboratory mice ideally suited to a wide variety of research areas.

In cardiovascular, pharmacological and toxicological research, accurate measurement of parameters relating to the circulatory system of laboratory animals is often required. Determination of heart rate, heart rate variability, and duration of PQ and QT intervals are based on electrocardiogram (ECG) recordings. However, obtaining reliable ECG curves as well as physiological data such as core body temperature in mice can be difficult using conventional measurement techniques, which require connecting sensors and lead wires to a restrained, tethered, or even anaesthetized animal. Data obtained in this fashion must be interpreted with caution, as it is well known that restraining and anesthesia can have a major artifactual influence on physiological parameters[1, 2].

Radiotelemetry enables data to be collected from conscious and untethered animals. Measurements can be conducted even in freely moving animals, and without requiring the investigator to be in the proximity of the animal. Thus, known sources of artifacts are avoided, and accurate and reliable measurements are assured. This methodology also reduces interanimal variability, thus reducing the number of animals used, rendering this technology the most humane method of monitoring physiological parameters in laboratory animals[3, 4]. Constant advancements in data acquisition technology and implant miniaturization mean that it is now possible to record physiological parameters and locomotor activity continuously and in realtime over longer periods such as hours, days or even weeks[3, 5].

Here, we describe a surgical technique for implantation of a commercially available telemetry transmitter used for continuous measurements of core body temperature, locomotor activity and biopotential (i.e. onelead ECG), from which heart rate, heart rate variability, and PQ and QT intervals can be established in freeroaming, untethered mice. We also present pre-operative procedures and protocols for post-operative intensive care and pain treatment that improve recovery, well-being and survival rates in implanted mice[5, 6].

Video Link

The video component of this article can be found at http://www.jove.com/video/3260/

Protocol

The animal experiment was approved by the Cantonal Veterinary Office (Zurich, Switzerland). Housing and experimental procedures were in accordance with Swiss Animal Protection law and conform to the European Directive on the Protection of Animals Used for Scientific Purposes (DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010).

1. Pre-operative considerations

1.1 Mice: housing requirements, general condition and health monitoring

It is recommended that mice delivered from vendors or transferred from external rodent colonies should arrive at the housing facility at least two weeks prior to surgery. This period should allow the animals to adapt to the new environment and facility-specific housing conditions. Mice, as social living animals, should be housed in compatible groups during this adaptation period. For monitoring an individual's level of food and water consumption, each mouse is housed singly from 3 days before surgery until 10 days after surgical transmitter implantation. The time line for establishing telemetric-transmitter-bearing mice is shown in Figure 1. It is crucial that the animals come to surgery in good health and condition. Therefore, before surgery, animals should be monitored once per day for 2-3 days concerning general condition (appearance, posture, spontaneous behavior) as well as for body weight, food and water consumption. These data are documented on a medical record (general

condition and health monitoring data sheet, Table 1) to establish individual baseline levels of general condition and overall health and wellbeing. Any animals showing symptoms of disease or impaired general condition before surgery should be excluded from the experiment.

1.2 Hair clipping at one day prior to surgery

The day prior to implantation, in order to shave the animals for surgery, mice are anesthetized briefly in a small (8x8x8cm) Perspex chamber using sevoflurane (8%) or isoflurane (5%) in pure oxygen (600 mL/min). After loss of the righting reflex, the mouse is taken out of the chamber and the anterior neck and abdominal hair is clipped with the animal lying in dorsal recumbence; anesthesia is maintained for approximately 5 minutes with a nose mask with sevoflurane 3-4% or isoflurane 1.5-3% in pure oxygen at a flow rate of 600 mL/min. After clipping the hair, the animals are allowed to awaken and are then brought back to their home cage.

2. Implantation

2.1 Operating environment, preparation of the telemetric transmitter

On the day of implantation, all procedures regarding transmitter preparation and surgery are carried out on a work bench with a laminar flow hood equipped with a surgical microscope. Aseptic conditions are assured by the use of autoclaved instruments and sterilized materials and by disinfecting the work bench[7]. Prior to implantation, the telemetric transmitters (ETA-F10, Data Sciences International, St. Paul, MN, USA) are first prepared. After removing from their sterile package, the leads of the transmitter are shortened to a length appropriate for the size of the mouse to be implanted. In the majority of adult outbred or inbred mice, the red electrode may be shortened to approximately 42 mm and the white/colourless electrode to a length of approximately 55 mm. Insulation tubing is removed from the distal (sensory) part of the leads: approximately 20 mm of tubing is removed from the red electrode, approximately 10 mm of tubing is removed from the white/colourless electrode. The distal part of each electrode (which is now without tubing) is formed into a loop by fixing the end with thin silk sutures (PERMA-Handseide, 6-0, Ethicon, Norderstedt, Germany). After preparing the electrodes, the transmitter is placed in warm sterile saline ready to be implanted when the animal is anesthetized and surgically prepared.

2.2 Anesthesia

At 5-10 minutes before induction of inhalation anesthesia, a mixture of midazolam (4 mg/kg) and fentanyl (0.04 mg/kg) are administered subcutaneously as premedication, thus providing sedation and pre-emptive analgesia. General inhalation anesthesia is induced by placing the animal in the induction chamber and introducing the volatile anesthetic agent (sevoflurane 8% or isoflurane 5% in pure oxygen 600 ml/min). When the animal shows loss of the righting reflex it is transferred to the work bench under the laminar flow hood, and placed in dorsal recumbence on a specially designed metal plate fitted with a nose mask and tubing from the anesthesia apparatus. Anesthesia is maintained by spontaneous breathing (sevoflurane 3-4% or isoflurane 1.5-3% in pure oxygen at a flow rate of 600 mL/min). During anesthesia, the animal's eyes are protected with ointment (Vitamin A, Baush & Lomb, Steinhausen, Switzerland). While lying on the metal plate the animal is warmed by the water-bath heated surface (39°C +/-1) of the work bench.

2.3 Surgery

The skin of the anterior neck and abdominal region is disinfected with 70% ethanol. A 1- to 1.5-cm-long incision in the skin is made from the lower thorax along the midline to the abdomen. The negative (white/colourless) lead is tunnelled subcutaneously from the thorax to the neck, where a small incision (≤0.5 cm) is made in the longitudinal direction. The skin and underlying tissues are prepared to make space for the fixation of the wire loop of the electrode. The wire loop is fixed between the muscles located to the right of the trachea, using two thin silk sutures (PERMA-Handseide, 6-0, Ethicon, Norderstedt, Germany). The wound in the neck is then closed with absorbable sutures (VICRYL 6-0, Ethicon, Norderstedt, Germany) in layers. The abdominal wall is then opened at the linea alba and the body of the telemetric transmitter is placed into the abdominal cavity of the mouse. The wire loop of the positive (red) electrode is sutured to the xiphoid process with silk sutures in such a way that it lies between the liver and the diaphragm in the left upper abdominal region (Figure 2). Then, the muscle layers of the abdominal region are closed with absorbable sutures (VICRYL 6-0, Ethicon, Norderstedt, Germany). Before finally closing the abdominal wall, a mixture of Sulfadoxin and Trimethoprim [(30 mg/kg and 6 mg/kg, respectively; dissolved in 1 mL of saline (0.9%) and at approximately body temperature (38-39°C)] is injected into the abdominal cavity for the purposes of anti-infective prophylaxis and to support fluid homeostasis. Finally, the skin of the abdominal region is restored with staples (Precise, 3 M Health Care, St. Paul, MN, USA).

3. Post-operative care

After completion of surgery and anesthesia, 0.1 mg/kg of buprenorphine (Temgesic, Essex Chemie AG, Lucerne, Switzerland) and 5 mg/kg of meloxicam (Metacam, Boehringer Ingelheim, Basel, Switzerland) is administered subcutaneously for pain treatment, and the animals are left on the warm (39°C +/-1) surface of the work bench to recover for approximately 2h. Together with pain relief (twice daily: buprenorphine, 0.1 mg/kg and meloxicam 5 mg/kg), supportive therapy consisting of 300 µL glucose (5%) and 300 µL saline (0.9%) warmed to body temperature, is applied subcutaneously twice daily for 4 days. For further recovery support, it is worthwhile providing the animals with an additional drinking bottle containing 15% glucose solution. During the recovery period of 4-10 days, it is recommended that the animals are kept warm. Therefore, in our case, the mice are housed in a warming cabinet (30°C +/- 1). Monitoring of general condition and body weight, as well as food and water consumption, is performed once daily according to the general condition and health monitoring data sheet (Table 1) for 10 days post-operatively. Humane endpoints, i.e. the sacrifice of an animal to avoid unnecessary suffering and pain if progression of recovery is unsatisfactory, are realised under the following conditions:

i. *If in poor general condition, i.e. the animal is substantially apathetic (no movement after being touched/pushed) and its body surface feels cold despite warming, the animal should be euthanatized immediately.*
ii. *If, on day 4 after transmitter implantation, the animal shows clear signs of apathy, is extremely aggressive or does not show any food intake, it should be euthanatized immediately.*
iii. *On day 8 after transmitter implantation, the animal has to display a clear increase in body weight in comparison to the preceding post-operative days. Moreover, it has to consume at least 80% of the pre-operative daily food intake. If one of these conditions is not met, the animal should be euthanatized immediately.*

At 10 days after implantation, the animal is transferred back to the animal room under standard housing conditions. Mice should be housed in compatible groups to allow social interaction and to prevent the adverse effects of long-term individual housing, which can have substantial

impacts on the read-out of subsequent experiments[8, 9]. Mice should have a period of at least 4 weeks convalescence after transmitter implantation before the first experiment is conducted and data acquisition begins.

4. Data acquisition

Data collection is initiated by touching the animal with a magnet, whereupon the transmitter is switched on. Dataquest A.R.T. Software (Data Sciences International, St. Paul, MN, USA) coordinates the detection, collection, analysis and graphical presentation (in the form of wave forms) of signals from one or more animals. The Acquisition program collects data signals sent to the computer from the converters and receivers via a Data Exchange Matrix (Data Sciences International). This program can either collect data for a specific length of time at regular intervals or sample continuously and save the data on the computer's hard drive. As the range and the quality of the emitted signal depends strongly on the material composition of the cage and surrounding equipment (e.g. metal vs. plastic), it is suggested that the receiver plate is placed as close to the animal as possible, e.g. under the animals' cage or above the experimental area, e.g. laboratory bench or treadmill. It is recommended that the correct configuration of the recording and data transmission system be checked by making a short examination of real-time measurements in continuous sampling mode. After the data have been gathered and stored, they can be plotted, listed and analysed for a variety of different parameters using the Analysis program. Details of the configuration of the recording system (e.g. defining the sampling modus), and analysis software (e.g. for heart rate variability parameters, PQ interval and QT interval established from biopotential/ECG curves) can be found in the manufacturer's manuals. Valuable hints for biometric planning and statistical methods useful for telemetric data acquisition and interpretation are published elsewhere[3].

5. Representative Results:

An overall scheme of the described procedure is shown in Figure 1. The position of the implanted transmitter, including the location of the electrodes for obtaining biopotentials from the heart (one-lead ECG) is shown in Figure 2. Examples of raw data from short term biopotential curves (one-lead ECG), and long-term heart rate, core body temperature and locomotor activity recordings of individual mice are given in Figure 3 and Figure 4, respectively. Figure 5 gives an example of published data from long-term measurements in groups of mice after an experiment. Several other parameters can be established from the biopotentials curves. Examples for presentation of heart rate variability parameters[5], QT interval and PQ interval[10, 11] are published elsewhere.

Table 1. General condition and health monitoring data sheet. Click here to download the sheet. This template facilitates monitoring of an individual mouse's general condition and health. Baseline examination of an animal's appearance, posture, and spontaneous behavior, as well as determination of body weight, and food and water consumption must be established before implantation surgery once per day for 3 days. Comparison of baseline determinations with those obtained daily for 10 days after surgery serve to assess the progression of post-operative recovery. In addition, post-operative care and pain treatment are well documented in the form of a medical record. Instructions on humane endpoints are given in order to facilitate decisions on whether a mouse should be sacrificed to prevent unnecessary pain and suffering if the animal does not meet the criteria for fast recovery after implantation.

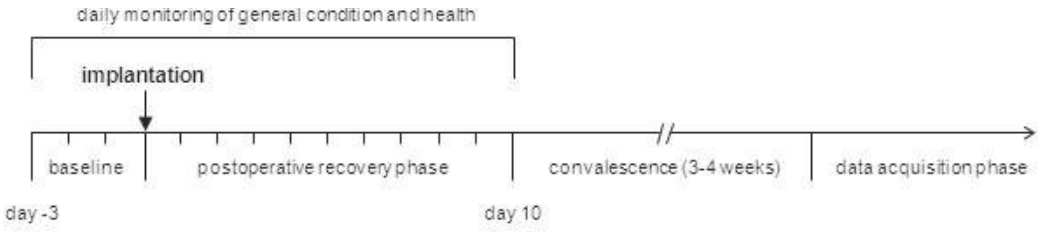

Figure 1. Schedule for establishing telemetric-transmitter-bearing mice. Chronological order of procedures relating to the implantation of a transmitter showing the time points at which a mouse can be used for experiments and data acquisition.

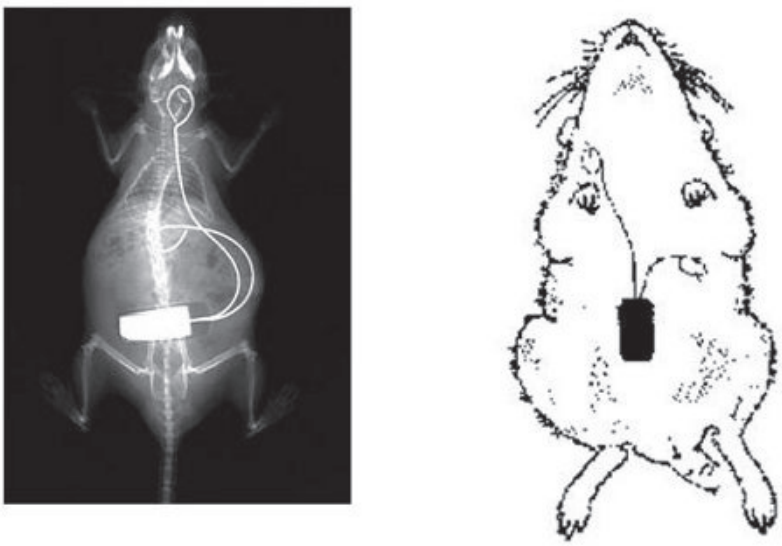

Figure 2. Radiograph/sketch showing location of the implanted telemetry transmitter. The body of the transmitter is positioned in the abdominal cavity. The positive lead is formed into a wire loop and fixed to the xiphoid process with sutures. The negative lead is tunneled subcutaneously

from the thorax to the neck and fixed as a wire loop between the muscles directly next to the trachea. The radiograph is taken from the authors' previous publication in Laboratory Animals[9].

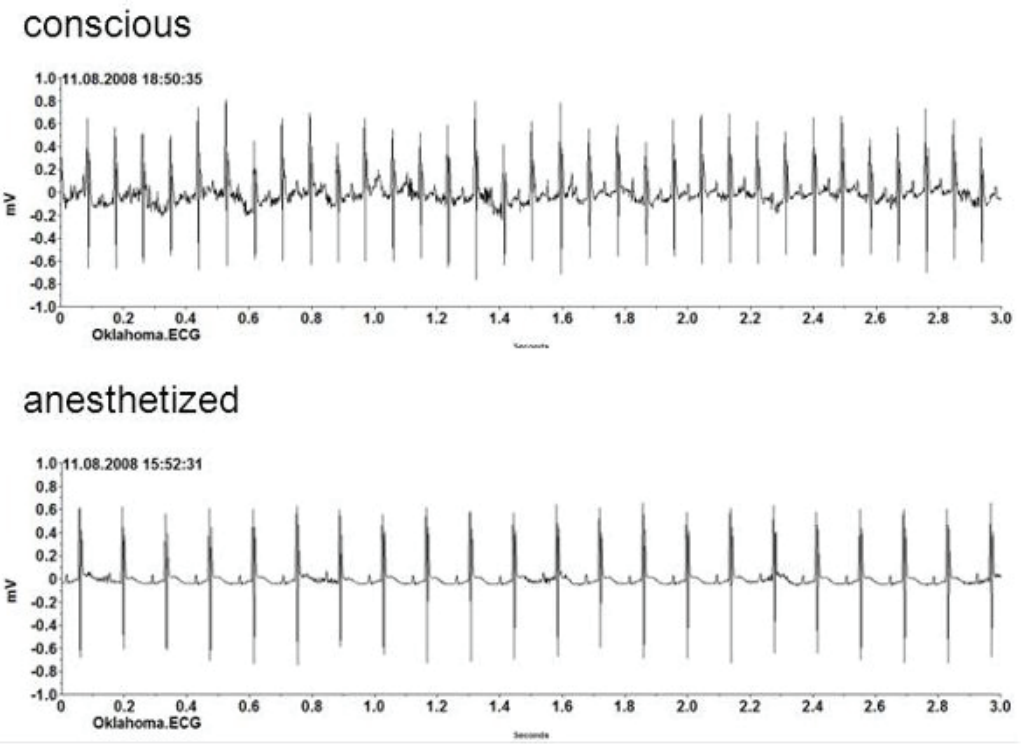

Figure 3. Biopotential curves. Raw printout of one-lead ECG curves from a conscious mouse and of the same animal under inhalation anesthesia with sevoflurane. Heart rate is calculated automatically by the telemetry system. The 3-second sequence recorded under anesthesia indicates a heart rate of 440 bpm. The curve recorded in the conscious mouse shows a heart rate of 660 bpm, which falls within the expected range for heart rate during moderate physical activities such as grooming or eating. From biopotential/one-lead ECG curves, heart rate variability parameters, interbeat interval, and PQ and QT intervals can be established with use of the manufacturer's software.

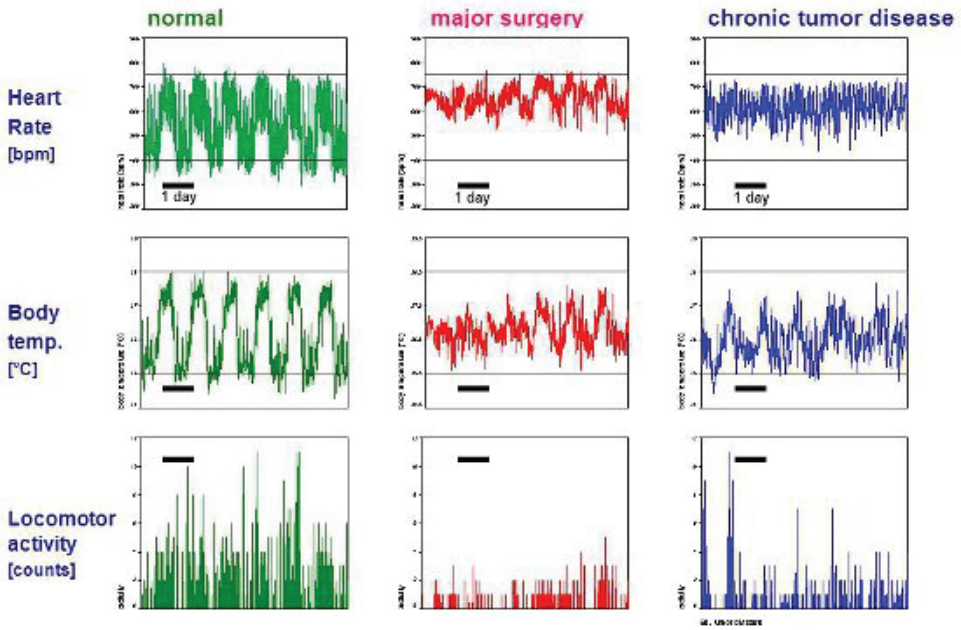

Figure 4. Raw data from long-term measurements in healthy and diseased mice. Heart rate (bpm), core body temperature (°C) and locomotor activity (counts) are measured while mice are housed individually in their home cage without any disturbance from man or experimental procedures. Heart rate is recorded for 30 seconds every 5 minutes (sampling frequency 1000 Hz). Core body temperature is sampled for 10 seconds every 5 minutes. Locomotor activity is recorded continuously and stored at 5 minute intervals. Five-minute data points are traced for 6.5 days. The telemetric measurements are recorded from three mice with differing bodily conditions. The healthy mouse shows a clear circadian rhythm with normal increases in physiological values and locomotor activity behavior during the dark (night) phase. In contrast, after major surgery, heart rate is increased, particularly in the daylight phase, and locomotor activity is depressed. The third mouse suffered from chronic tumor disease-its circadian rhythm of heart rate and core body temperature appears flattened, and locomotor activity is diminished. Representative data of heart rate measurements (normal values and after major surgery) are taken from the authors' previous publication in Altex [12].

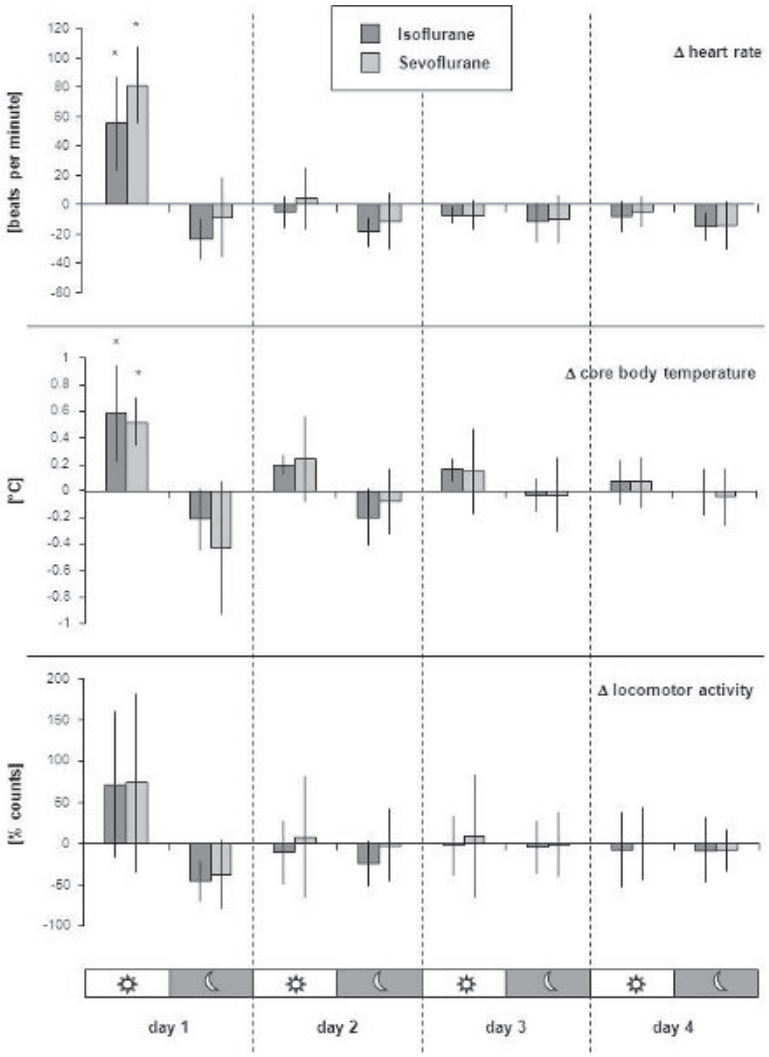

Figure 5. Example of presentation of results from long-term telemetry measurements after an experiment. The figure is taken from the authors' previous publication in Laboratory Animals[1]. As an exemplary experiment, a 50-minute isoflurane or sevoflurane anesthesia was performed. The long-term impact of the anesthetics on heart rate, core body temperature and locomotor activity after the animals were awake was compared. Using 16 transmitter-implanted mice, telemetric data were recorded in eight mice per anesthetic while the animals were single-housed and allowed to roam freely in their home cages. For analysis of long-term postanesthetic effects, we took into account that values vary greatly during a 24-h cycle since mice are active mainly at night. Therefore, the means of the telemetric values for each animal were calculated separately for the night (12 h dark) and day (12 h light) phases. An individual's normal values were established by calculating means from the three days prior to anesthesia. For each day after anesthesia, the mean of the dark and light phase was compared with the individual's normal values, resulting in delta values. Thus, delta values represent deviation from normal values (established prior to anesthesia) at the corresponding 12 h day and night time. Columns represent the mean from eight mice; bars indicate standard deviation. Asterisks indicate significance at P ≤0.05 (One-way analysis of variance for comparison of group means at each of the four days after anesthesia with normal values).

Discussion

Radiotelemetry is a powerful alternative to conventional methods of measurement of physiological parameters in biomedical research. High-quality telemetry systems consisting of implantable transmitters, receivers and data acquisition and analysis hardware and software are now commercially available, even for animals as small as mice. Telemetry represents the only technique currently available for data collection from unrestrained, freely moving mice. By using this method, it is now possible to gather data continuously and/or for longer periods of time from animals residing in their own familiar environment, thus minimizing the stress to the animals and consequent experimental artifacts. The form and position of the leads has been optimized in order to obtain signals even during fast movements (e.g. struggling, running, fighting) or in an upright posture[9]. Thus, accurate measurements can be obtained during experiments, e.g. during anesthesia, stress induction, while running on a treadmill, during behavioral experiments, during infection experiments, and many other experimental situations.

However, in order to obtain reliable, reproducible and artifact-free data, it is crucial to exclude environmental influences, and we draw particular attention to the importance of standardized conditions. It is recommended that the room is isolated from electronic and acoustic noise, including ultrasonic sound, to which mice are particularly sensitive. In addition, no disturbances, such as visitors or unrelated experimental procedures, should be allowed when conducting measurements. To avoid interfering influences (particularly in case of home cage measurements), all necessary husbandry procedures should be completed in the room before starting each measurement. In addition, the housing of mice-particularly if males are used-in groups or individually can have an impact on the measurements and must be considered when planning experiments[9]. Also, the mice must be healthy and free of murine pathogens, since latent or manifest infections, as well as diseases or any other health impairments, can have considerable influence on physiological parameters and activity behavior. Accordingly, mice should recover fully after implantation and be given sufficient time to adapt to bearing the transmitter before starting any experiments.

Data collection by radiotelemetry in mice requires preliminary surgical implantation of the telemetry transmitter. This should be performed only by trained personnel with surgical skills in order to minimize tissue trauma and subsequent pain and distress. For experimenters holding basic or even advanced (micro-) surgical skills, it is recommended to perform the first trials in fresh mouse cadavers using training implants (i.e., dummies, provided by the manufacturer) to establish the procedures and become familiar with the specifics of this kind of surgery. After such training, most experimenters would be capable to implant this type of transmitters with success and would reach a useful proficiency after a few implantations.

Aseptic conditions should be maintained during surgery to keep the microbiological burden and the risk of infections low. However, complete sterility cannot be provided because of some specific, sterility conflicting conditions in mice (e.g., cooling effect of extensive hair clipping and disinfection, impracticality of bandages to protect the wounds). Thus, anti-infective prophylaxis is administered during the implantation. Well tailored analgesic treatment and a clearly defined monitoring plan as well as adequate post-operative care play a crucial role in the satisfactory outcome of the experiment.

Overall, the surgical implantation of a telemetric transmitter in mice will be stressful for the animal. In particular, if genetic modification in specific mouse lines influences the phenotype and impairs the animals' bodily condition, complications in the peri-operative time frame and increased death rates after implantation might be a risk. To avoid unnecessary suffering, individuals exhibiting unsatisfactory recovery or prolonged convalescence should be released from the experiment and sacrificed before reaching a moribund stage. For this purpose, a data sheet (Table 1: general condition and health monitoring data sheet) facilitating the systematic monitoring of critical symptoms and providing advice on humane endpoints has been established. Thus, recovery is documented in the style of a medical record or a laboratory journal, which makes the conducting of this methodology (i.e. implantation procedure and post-operative recovery) transparent to the relevant authorities and animal welfare bodies responsible for animal experimentation (e.g., IACUC).

Disclosures

No conflicts of interest declared.

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Original Article

Isoflurane and sevoflurane provide equally effective anaesthesia in laboratory mice

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Abstract

Isoflurane is currently the most common volatile anaesthetic used in laboratory mice, whereas in human medicine the more modern sevoflurane is often used for inhalation anaesthesia. This study aimed to characterize and compare the clinical properties of both anaesthetics for inhalation anaesthesia in mice. In an approach mirroring routine laboratory conditions (spontaneous breathing, gas supply via nose mask, preventing hypothermia by a warming mat) a 50 min anaesthesia was performed. Anaesthetics were administered in oxygen as carrier gas at standardized dosages of 1.5 minimum alveolar concentrations, which was 2.8% for isoflurane and 4.9% for sevoflurane. Both induction and recovery from anaesthesia proceeded quickly, within 1–2 min. During anaesthesia, all reflex testing was negative and no serious impairment of vital functions was found; all animals survived. The most prominent side-effect during anaesthesia was respiratory depression with hypercapnia, acidosis and a marked decrease in respiration rate. Under anaesthesia, heart rate and core body temperature remained within the normal range, but were significantly increased for 12 h after anaesthesia. Locomotor activity, daily food and water consumption and body weight progression showed no abnormalities after anaesthesia. No significant difference was found between the two anaesthetics. In conclusion, isoflurane and sevoflurane provided an equally reliable anaesthesia in laboratory mice.

Keywords: Isoflurane, sevoflurane, anaesthesia, mouse, mice

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Rodents are usually anaesthetized by injection of hypnotic, analgesic and muscle relaxant liquid agents.[1] Since continuous intravenous, target-controlled infusion, or the so-called total intravenous anaesthesia, with short-acting drugs such as propofol (e.g. ref. 2), is hard to master in mice, the intraperitoneal or subcutaneous application route is normally chosen in this species.[3–5] Although it would seem easy and practical to induce general anaesthesia with an injection of a single (e.g. pentobarbital) or mixed (e.g. ketamine/xylazine, medetomidine/midazolam/fentanyl) long-acting drug(s), this type of anaesthesia is hard to control. Once the initial dose has been administered, the duration and depth of anaesthesia cannot be adjusted to the specifics of the mouse (strain, age, gender, individual variation, etc.) or the surrounding conditions (time of day, housing conditions, etc.), all of which influence the animals' response to the anaesthetic.[6–10] Thus, despite prior dosage testing, managing injection anaesthesia often remains difficult, i.e. on the one hand anaesthesia is shallow in some individuals, and on the other, the death rate can be unexpectedly high.[3,4,11]

Such failures are rarely encountered with inhalation anaesthesia. Modern, commercially available volatile anaesthetics such as isoflurane, sevoflurane, desflurane and others are vaporized in dedicated vaporizers, added to a carrier gas and supplied to the animal via the respiratory tract. Due to their low blood:gas partition coefficients, these compounds provide rapid induction of anaesthesia, are short-acting and are removed from the body in a short time, mostly by respiration.[12] The dosage can be adapted easily and can be titrated to effect for an individual animal. Thus, provided that the animals' vital functions and depth of anaesthesia are monitored, cases of death are unusual because the course of anaesthesia can be easily controlled. Thus, recently developed volatile anaesthetics are used increasingly in laboratory rodents,[1,13] especially

since ready-to-use inhalation anaesthesia devices tailored for small rodents are commercially available. The most up-to-date anaesthesia equipment[14–16] normally includes active scavenging systems to prevent the release of waste gas, which is mandatory for protecting personnel and which has been a problem in the past.[17–20]

The most common and well-known volatile anaesthetic in laboratory rodents is isoflurane.[1] Sevoflurane, a more modern inhalation anaesthetic, is used in human medicine,[21] but is uncommon in veterinary medicine due to its higher cost. To date, the clinical impacts of isoflurane and sevoflurane have been described mostly in man or in animal species other than mice. This led us to investigate the possible advantageous properties and drawbacks of isoflurane and sevoflurane anaesthesia in laboratory mice. These two substances were compared in a practical setting for their effects during and after anaesthesia from a clinical viewpoint, with the aim of determining their impact on animal physiology and general post-anaesthetic condition.

Materials and methods

Animals

Sixty-four, six-week-old female C57BL/6J mice were obtained from our in-house breeding colony. The mice were free of all viral, bacterial and parasitic pathogens listed in FELASA recommendations.[22] Health status was monitored by a sentinel programme throughout the experiments.

Animals were kept in type 3 open-top plastic cages (425 mm × 266 mm × 150 mm, floor area 820 cm^2) with autoclaved aspen bedding (80–90 g/cage) (LTE E-001 Abedd, Indulab, Gams, Switzerland). Autoclaved hay (8–12 g/cage) and two Nestlets™ (each 5 × 5 cm), consisting of cotton fibres (Indulab), were provided as nesting materials. A standard cardbox house (Ketchum Manufacturing, Brockville, Canada) was provided. Animals were fed a pelleted mouse diet (Kliba No. 3431, Provimi Kliba, Kaiseraugst, Switzerland) *ad libitum* and had unrestricted access to sterilized drinking water. The light/dark cycle in the room consisted of 12/12 h with artificial light (approximately 40 lx in the cage) from 3:00 to 15:00 h. The temperature was $21 \pm 1^{\circ}C$, with a relative humidity of $50 \pm 5\%$, with 15 complete changes of filtered air per hour (HEPA H 14 filter); the air pressure was controlled at 50 Pa.

Mice were housed in groups, except during the four days before and four days after anaesthesia, when they were housed individually. The first day of single housing served for adaptation to the change in housing conditions; from the second day onwards, the individuals' normal values for heart rate, core body temperature, locomotor activity, body weight, food and water consumption were recorded. To avoid interfering influences, all necessary husbandry and management procedures were completed in the room before starting single housing of mice, and disturbances (e.g. visitors or unrelated experimental procedures) were not allowed. The animal room was insulated to prevent electronic noise.

This study was approved by the Cantonal Veterinary Office (Zurich, Switzerland) under the licence number 111/2007. Housing and experimental procedures were in accordance with Swiss animal protection law and conform to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe No. 123, Strasbourg, 1985).

Preliminary transmitter implantation

Prior to the experiments, at age 10 weeks, 16 mice were instrumented with telemetric transmitters. TA10ETA-F20 transmitters (Data Sciences International, St Paul, MN, USA) – which measure heart rate, core body temperature and locomotor activity in freely moving mice – were implanted as previously described in detail.[23] Briefly, under anaesthesia with sevoflurane (Sevorane™, Abbott, Baar, Switzerland), the transmitter body was implanted in the abdomen under aseptic conditions. One wired loop electrode was fixed with silk sutures between the muscles located to the right of the trachea, whereas the other wired loop lead was sutured to the xiphoid process. Muscle layers and skin were closed with resorbable sutures. Postoperatively, buprenorphine (Temgesic™, Reckitt and Colman Products Ltd, Hull, UK) was given at a dose of 0.1 mg/kg body weight, injected subcutaneously twice per day for four days.[24] After transmitter implantation, the mice had a period of six weeks convalescence before the first experiment.

Experimental setting

All experiments were conducted when the mice were aged 16–36 weeks, with body weights ranging from 25 to 30 g. All experiments and weighing procedures were carried out between 15:00 and 18:00 h. Anaesthesia was performed in a separated operating area within the animal room.

Anaesthesia was provided by a commercially available rodent inhalation anaesthesia apparatus (Provet, Lyssach, Switzerland), which was equipped with interchangeable vaporizers for isoflurane (Ohmeda Isotec 5, Abbott) and sevoflurane (Ohmeda Sevotec 5, Abbott). As carrier gas, 100% oxygen was used at a flow rate of 400 mL/min. The anaesthetic gas was introduced into the nose mask (inner diameter 1.2 cm) through a thin tube (outer diameter 0.4 cm, inner diameter 0.3 cm). The opening of the thin tube was at a distance of exactly 0.5 cm from the latex membrane, which had a hole in the centre that fit around the nose of the mouse. The nose of the mouse was placed 2 mm in front of the opening of the inner tube. The nose mask merged into a thick outer tube (surrounding the thin inner tube), which allowed waste anaesthetic gas to be eliminated from the nose mask by a pump-driven filter system (flow rate 400 mL/min). The same principle was utilized for the induction chamber (inflow and outflow 400 mL/min). The concentrations of anaesthetic gases in the nose mask (at 2 mm in front of the opening of the inner tube) and in the induction chamber were measured at the beginning of anaesthesia and then every 5 min using a side stream monitoring device employing infrared

technology (Datex-Ohmeda AS/3, Anandic Medical, Deissenhofen, Switzerland). The device was calibrated just before use using the proprietary standard reference gas supplied by the manufacturer.

Determination of minimum alveolar concentration

Forty-eight non-transmitter-implanted mice underwent anaesthesia three to four times in order to standardize anaesthesia by establishing minimum alveolar concentration. Care was taken that the animals had a break of at least two weeks between tests.

Minimum alveolar concentration was determined with a protocol modified from published methods.[25–27] Briefly, after inducing anaesthesia in the chamber for 2 min at maximum concentration of anaesthetic gases (5% isoflurane, 8% sevoflurane), the mouse was taken out of the chamber and placed in dorsal recumbency on a warmed mat (see below). Anaesthetic gas was then applied at the desired concentration via a nose mask, with the mouse breathing spontaneously. After an equilibration time of 10 min, painful stimuli in the form of pinching the tail, the interdigital webbing (pedal withdrawal reflex), the abdominal skin or neck skin were applied every 5 min for the next 30 min. All stimuli were induced by the same investigator by using blunt forceps containing a spacer between its arms. The motor response to a painful stimulus was evaluated as positive or negative, i.e. whether a motor response was visible or not.

Using this protocol, rough minimum alveolar concentration was first estimated within the concentration window (1–3% and 2–4% for isoflurane and sevoflurane, respectively) in which we empirically expected the minimum alveolar concentration to lie. Concentrations were graded in 0.25% steps and 10 animals per concentration were used.

Finally, four concentrations were chosen, and 25 animals per concentration were then tested. Minimum alveolar concentration was then calculated as the mean of the two partial pressures bracketing the response or lack of response in our tested population.

Anaesthesia experiments

Anaesthesia was induced by placing the mouse in a clear Perspex induction chamber ($8 \times 8 \times 8$ cm, volume 512 mL), into which either 5% isoflurane (Isoflo^{TM}, Abbot) or 8% sevoflurane (Sevorane^{TM}, Abbott) was then introduced. After 2 min, the animal was quickly transferred to a nose mask, where anaesthesia was maintained with 2.8% isoflurane or 4.9% sevoflurane, equivalent to 1.5 minimum alveolar concentrations (as established above). Mice breathed spontaneously while lying in dorsal recumbency on a water-filled warming mat (Gaymar, TP500, Orchard Park, NY, USA) set at $38 \pm 1^{\circ}$C.

Tail pinch, pedal withdrawal and abdominal skin pinch reflexes were applied at 5 min intervals. All reflex tests were induced by the same investigator by using blunt forceps containing a spacer between its arms. The reflex tests were registered as positive or negative, i.e. whether any motor response was observable or not. Respiration rate was counted from the movement of the thorax wall.

Anaesthesia was stopped after 50 min by removing the nose from the mask and letting the mouse breathe room air. Two minutes later, when the mice had righted themselves from dorsal to ventral recumbency and were able to move, they were placed back in their home cage.

Telemetric data acquisition and analysis

Using the 16 transmitter-implanted mice, telemetric data were recorded in eight mice per anaesthetic. Mice were allocated randomly to the two groups. Telemetric data were recorded with the Dataquest LabPRO program (Data Sciences International). Data collection was initiated by switching on the transmitter with a magnet. Data acquisition started three days before anaesthesia and continued for the four days following anaesthesia.

To establish normal values (3 days before anaesthesia) and to investigate the postanaesthetic effects (4 days following anaesthesia), heart rate and core body temperature were measured every 5 min for 30 and 10 s, respectively. Locomotor activity was recorded continuously and stored at 5 min intervals.

To estimate the acute effects of anaesthesia (i.e. data measured during the 50 min anaesthesia experiment), heart rate and core body temperature were recorded for 4 s every 15 s (four measuring points of 4 s/min) while administering anaesthesia. From these data, the mean values of heart rate and core body temperature were calculated for each minute for each individual. Normal values represent means from the time period 15:00–18:00 h (i.e. the congruent time frame in which anaesthesia was carried out) during the three days prior to the experiment.

For analysis of long-term postanaesthetic effects, we took into account that values vary greatly during a 24 h cycle since mice are active mainly at night. Therefore, the means of the telemetric values for each animal were calculated separately for the night (12 h dark) and day (12 h light) phases. An individual's normal values were established by calculating means from the three days prior to anaesthesia. For each day after anaesthesia, the mean of the dark and light phase was compared with the individual's normal values, resulting in delta values.

Changes in body weight, and food and water intake

Body weight progression and daily food and water consumption were established from transmitter-implanted mice for three days before and three days after anaesthesia. Weights (animal, food pellets, water bottle) were recorded with a precision balance (PR 2003 Delta Range, Mettler-Toledo AG, Greifensee, Switzerland), especially adjusted for use with moving animals. Body weights recorded in transmitter-bearing mice were corrected to take into account the weight of the transmitter (3.6 g). The mean normal weights (from 3 consecutive daily measurements prior to the experiment) were calculated for each mouse, and compared with the weights recorded on each of the three days afterwards.

Acid–base balance and blood gas concentration

Three to four weeks after minimum alveolar concentration determination, the same 48 non-transmitter-implanted mice were used to obtain arterial blood with which to assess the acute side-effects of the anaesthetics used on respiration and acid–base balance.

Arterial blood was taken under anaesthesia at time points 10, 30 and 50 min of anaesthesia from eight mice per anaesthetic and time point. Following incision of the anterior neck, dissection of the right common carotid artery and cutting a small hole in the artery using a fine-bladed pair of scissors, arterial blood was collected in a heparinized syringe. Acid–base balance (pH), partial pressure of carbon dioxide (pCO_2, mmHg) and standard bicarbonate (HCO_3, mmol/L) were determined immediately using a blood gas analyser (AVL Compact 3, AVL List, Graz, Austria). These animals died immediately from the subsequent rapid loss of blood under anaesthesia. The normal values of pH, pCO_2 and HCO_3 used for comparison had been established previously from the arterial blood of 20 HanRcc:NMRI mice of similar age as those used in the present study.[4]

Statistical analysis

All data are presented as mean $\pm$ standard deviation. Statistical analysis using SPSS for Windows, version 13.0 was carried out to validate the results of the postanaesthetic effects of isoflurane and sevoflurane. One-way analysis of variance was performed to compare group means of heart rate, core body temperature and locomotor activity at each of the four days after anaesthesia in both anaesthetics with normal values. *Post hoc* analysis with Bonferroni tests was carried out to identify significant differences between groups; P values ≤ 0.05 were considered significant.

Results

Minimum alveolar concentration

Mean minimum alveolar concentration was established as 1.85% ($\pm$0.15%) for isoflurane and 3.25% ($\pm$0.14%) for sevoflurane in the adult female C57BL/6J mice used here. All anaesthesia experiments were conducted with 2.8% isoflurane or 4.9% sevoflurane, under which none of the mice showed a motor response to testing of the pedal withdrawal reflex, tail pinch or abdominal skin pinch.

Acute effects of anaesthesia

All mice were clearly immobilized within one minute after placing in the induction chamber. Monitoring of heart rate, core body temperature and respiration rate during anaesthesia revealed no deviation from normal values in heart rate and core body temperature. In contrast, the respiration rate decreased markedly below the normal values of the resting mouse (Figure 1).

Acid–base balance and blood gas measurement in the arterial blood showed acidosis (i.e. decrease of pH) and hypercapnia (i.e. increase of pCO_2) at 10, 30 and 50 min of anaesthesia (Figure 2).

When anaesthesia was completed, i.e. when the nose mask was removed, animals showed increasing respiration rate and muscle rigour within one minute. Mice turned to sternal recumbency and showed spontaneous movement at 1–2 min after anaesthesia was withdrawn.

Postanaesthetic effects

Telemetric measurements revealed a significant increase in heart rate and core body temperature for 0–12 h after anaesthesia compared with normal values (i.e. before anaesthesia) with both anaesthetics. In this time frame, locomotor activity also showed a tendency to increase, but this was not significant for either anaesthetic (Figure 3). Statistical comparison of isoflurane versus sevoflurane regarding their long-term effects on heart rate, core body temperature and locomotor activity revealed no difference between these two anaesthetics.

Body weight with both anaesthetics was constant in the postanaesthetic phase, i.e. mean body weights varied with $\leq$0.5% ($\pm$0.01%) compared with the normal body weight before anaesthesia. The mean daily food intake showed a decrease of 10% ($\pm$0.2%) at the first postanaesthetic day in both anaesthetics. At the second postanaesthetic day, mean food consumption was almost unchanged, with a decrease of 1.5% ($\pm$0.2%) in both anaesthetics. At the third postanaesthetic day, food intake was decreased with 5% ($\pm$0.1%) in isoflurane and 9% ($\pm$0.1%) in sevoflurane. Water consumption showed high interindividual variability. The mean water consumption in both anaesthetics showed an increase of 6% ($\pm$19% for isoflurane; $\pm$25% for sevoflurane) at the first day after anaesthesia. Mean water intake ranged from a decrease of 1.5% to an increase of 8.7%, with standard deviations ranging from 12% to 19% at the second and third postanaesthetic days in both anaesthetics. The alterations in body weight, food and water intake were not statistically significant.

Discussion

Prior to these investigations, the anaesthetic procedure and dosage of anaesthetics were standardized based on the specifics of the equipment (e.g. calibrating the gas concentration) and mouse population (e.g. strain, age, gender) used. Therefore, minimum alveolar concentrations were established following the widely accepted method of determining the concentration of anaesthetic gas at which 50% of the animals fail to respond with purposeful movements to the testing of reflexes. The minimum alveolar concentration determined for sevoflurane in female C57BL/6J mice was almost identical to that described for male outbred mice (3.25% in female C57BL/6J versus 3.22% in male CD-1).[28] The minimum alveolar concentration is known to vary considerably between mouse strains,[26] but other factors such as age and gender can also influence anaesthetic potency. Gender differences may explain why the minimum alveolar concentration for isoflurane was found to be higher (1.85%)

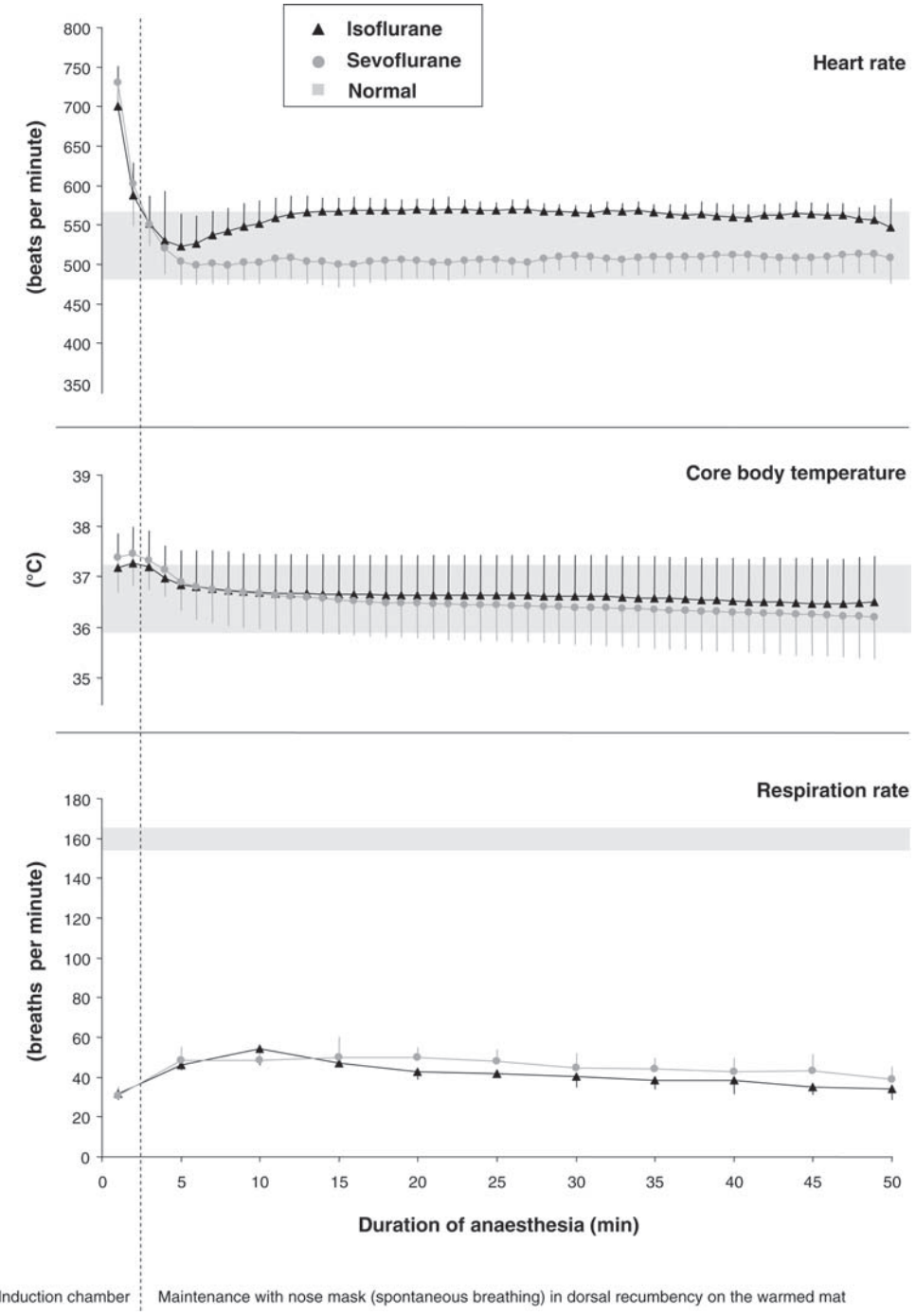

Figure 1 Heart rate, core body temperature and respiration rate during 50 min of anaesthesia with isoflurane or sevoflurane. The grey areas indicate normal values at the corresponding time of day in conscious animals. Data represent the mean values of eight mice; bars represent standard deviation

than values described in the literature for C57BL/6J mice (1.30%[27] and 1.34%[29]). In former reports, male mice[27] or a mixture of both sexes[29] were used, whereas in our experiments only female mice were investigated. On the other hand, the suggestion that anaesthetic requirements decrease with age, and literature reports of lower minimum alveolar concentrations in younger mice (7–9 weeks[27], 6–12 weeks[29]) are in contrast to the 0.5% increase found in our study, in which older mice (16–36 weeks) were used. However, when comparing minimum alveolar concentrations established in different laboratories, technical aspects of how the gas was provided must be considered. The easy-to-use open anaesthesia system used here might include an uncertainty in the absolute values of the gas concentration measurements.

After preliminary standardization of the dosages by establishing the minimum alveolar concentrations, the anaesthetics were then compared with dosages of isoflurane and sevoflurane representing 1.5 minimum alveolar concentrations. For this dosage it is generally postulated that 99.9%

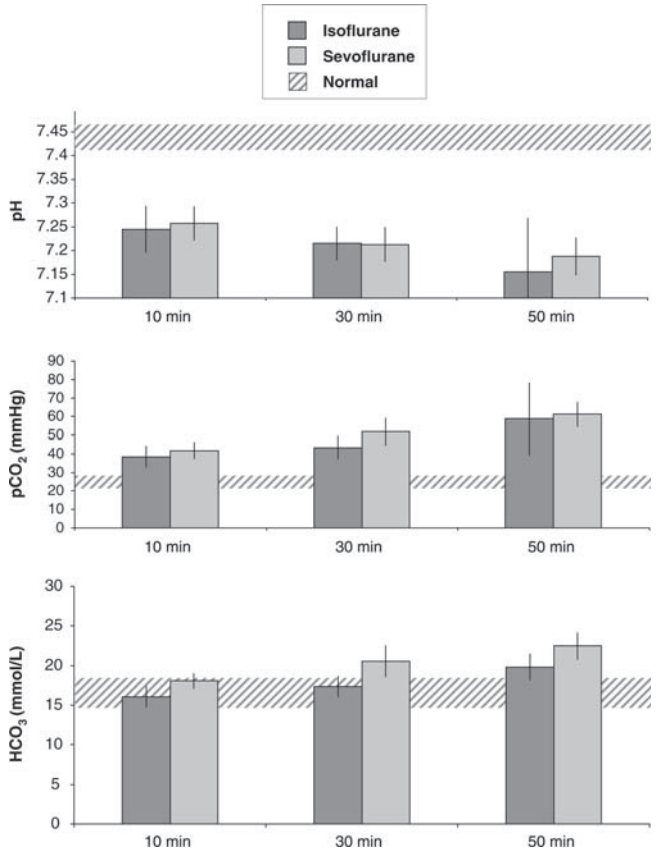

Figure 2 Acid–base balance (pH), partial pressure of carbon dioxide (pCO_2) and standard bicarbonate (HCO_3) in arterial blood at 10, 30 and 50 min of anaesthesia. Hatched areas indicate the normal values established in a previous study.[4] Data represent the mean values of eight mice; bars indicate standard deviation

of animals will not react to painful stimuli,[30,31] i.e. that the animals have reached surgical tolerance. However, since analgesia was not proved with sophisticated methods such as measurements of the reaction in heart rate, blood pressure or respiration upon a substantially painful stimulus (e.g. skin incision), surgical tolerance cannot be definitively claimed from our study. However, motor reflex responses were suppressed in all animals and none died from the 50 min inhalation anaesthesia with 1.5 minimum alveolar concentrations (i.e. 2.8% isoflurane or 4.9% sevoflurane).

In the early induction phase of anaesthesia, heart rate peaked at the upper normal level of 700–800 beats per minute (bpm), which we suggest as a normal reaction to removing the animal from its cage and placing it in a foreign environment. During anaesthesia, heart rate was stable within the normal values of the resting mouse (490–550 bpm). This was in agreement with recent publications indicating only slight depression of heart rate for isoflurane anaesthesia.[13,32–35]

During 50 min of inhalation anaesthesia, core body temperature could be maintained with a simple, water-bath-driven warming mat. That core body temperature falls due to any kind of anaesthesia is well known, and mice are especially sensitive to hypothermia due to their small size and high body surface area (e.g. a drop to 30–31°C was shown following isoflurane anaesthesia in mice[35]). Obviously, such hypothermia should be prevented, because it influences physiology and the course of anaesthesia and can ultimately lead to the death of the animal. Thus, warming the animal has been common practice for years, and is particularly worthwhile in long-term anaesthesia in mice.[36–38]

In contrast to the almost normal levels of heart rate and core body temperature, respiratory depression, as evidenced by decreased respiration rates (far below the values of the resting mouse), marked hypercapnia and acidosis, were seen to an equal extent with both isoflurane and sevoflurane anaesthesia. Hypercapnia and acidosis associated with isoflurane has also been found by others,[13] but was less intense than with injection anaesthesia with pentobarbital.[34] Other studies also report a marked decrease in respiration rate upon isoflurane anaesthesia, but values reported in the literature[36,38] were not as low as found here with both anaesthetics.

Thus, respiratory depression was the major adverse effect observed with both isoflurane and sevoflane. In general, the influence of inhalational anaesthetics on respiratory function is well known. By inhibiting the control systems of respiration (e.g. feedback control of central respiratory centres, various chemoreceptors, pulmonary reflexes and neuronal input) inhalational anaesthetics alter oxygen supply and CO_2 elimination.[39] This is mirrored by aberrations in arterial blood gas levels (e.g. increase of pCO_2, decrease of pO_2) and thus hampers the ability of the organism to maintain cellular homeostasis. Respiratory depression is often accompanied by acid–base imbalance (e.g. alterations of pH and HCO_3), and changes in the depth and frequency of respiration. Since respiratory depression is the most probable emergency situation when using isoflurane or sevoflurane anaesthesia, it may be useful to monitor the respiration rate as an indirect indicator of impaired respiration and thus prevent fatal outcomes under routine conditions. In addition, administering oxygen instead of room air in the recovery phase (i.e. after the volatile anaesthetic ceased) may be beneficial for animals with impaired respiration.

The time required for induction and recovery were almost the same for both anaesthetics (1–2 min). The short recovery time from both inhalational anaesthetics should be highlighted as a distinct advantage compared with injection anaesthesia. The benefits of fast recovery include reducing postoperative complications associated with prolonged inability to correct physiological impairment (e.g. hypothermia, hypoglycaemia, dehydration) that may induce suffering and hamper the rapid return of the animal to its normal state.

Heart rate and core body temperature increased significantly in the 12 h following the 50 min anaesthesia. Locomotor activity showed a tendency to increase only in the first hours after anaesthesia, suggesting that physical activity is not the reason for the elevated heart rate and core body temperature. Alterations in body weight progression as well as daily food and water consumption are known to hint at pain, distress or impaired wellbeing in laboratory animals.[40,41] Postanaesthetic determination of these indices revealed no relevant aberrations, indicating the negligible impact of a 50 min anaesthesia with isoflurane or sevoflurane on body weight, food and water

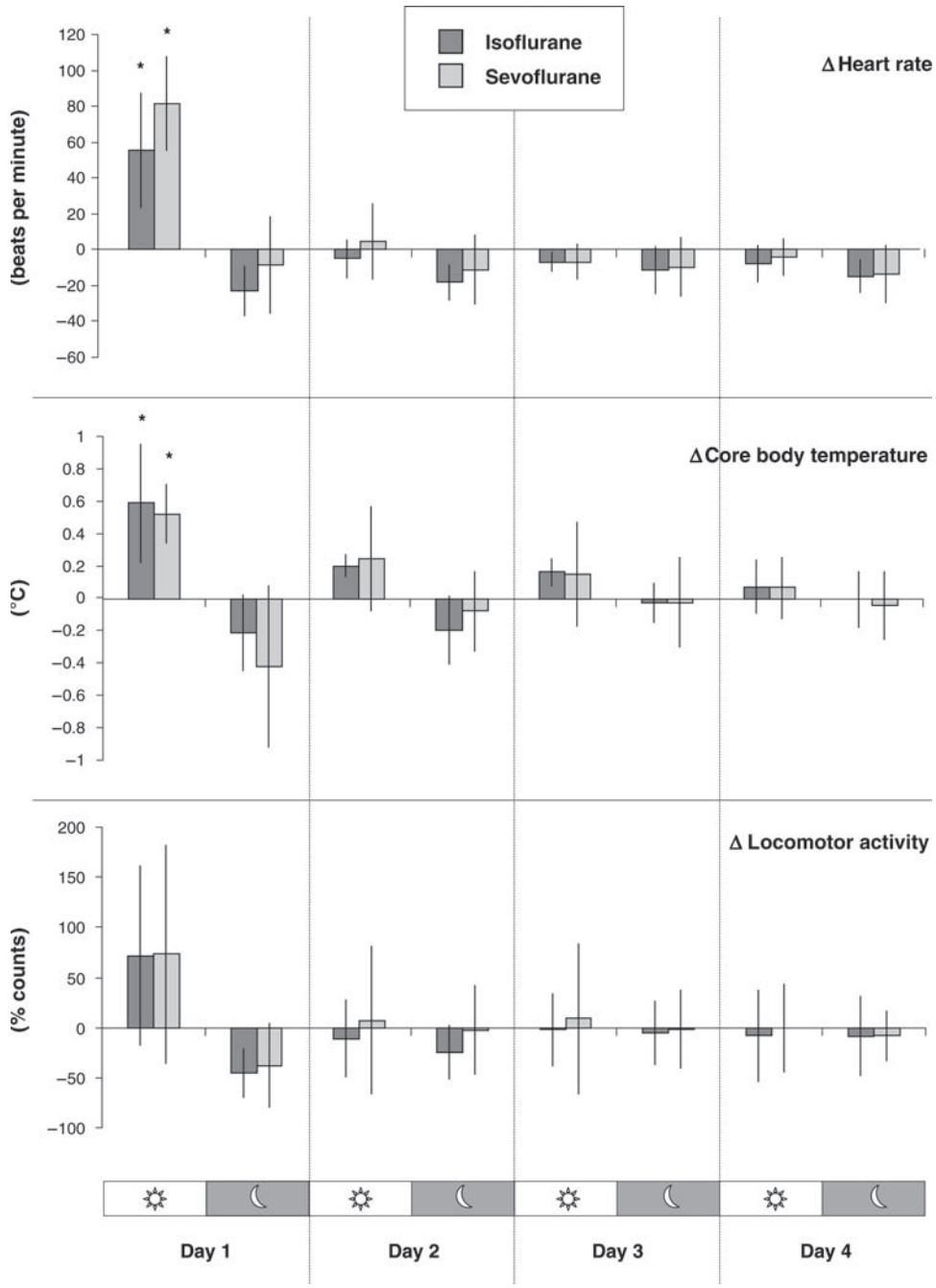

Figure 3 Postanaesthetic measurements of the impact of isoflurane and sevoflurane on heart rate, core body temperature and locomotor activity. Delta (Δ) values represent deviation from normal values (established prior to anaesthesia) at the corresponding 12 h day and night time. Data represent the mean from eight mice; bars indicate standard deviation. Asterisks indicate significance at $P \leq 0.05$

intake. Although it is unclear why the postanaesthetic elevation in heart rate and core body temperature occurred, it appeared to be of no long-term detriment to the animals.

Considering the effects of isoflurane and sevoflurane on physiology and behaviour, we found that both agents exerted a similar impact on the normal state established in the unanaesthetized mouse. Whereas several publications describe the usefulness of isoflurane, there is limited description of sevoflurane in mice in the literature. Henke and co-authors[36] compared induction and recovery times, and respiration rates of sevoflurane with those of isoflurane in the gerbil. Although they found prolonged recovery from isoflurane compared with sevoflurane, they concluded that there is no overall preference for one of these two volatile anaesthetics over the other. Another study compared blood glucose and some specific parameters required in functional PET investigations in mice; sevoflurane was considered superior compared with isoflurane, and the former was consequently recommended for physiological imaging by Flores and co-authors.[42] From our results, neither anaesthetic was clearly superior over the other.

In summary, we conducted inhalation anaesthesia in a routine, cost-effective setting, using commercially available equipment. The anaesthesia experiments were standardized

by establishing minimum alveolar concentrations, i.e. the dosage was adjusted to the characteristics of the animals used (female C57BL/J mice, aged 16–36 weeks). Both volatile anaesthetics tested showed short induction and recovery times in an easy-to-manage, standard inhalation anaesthesia procedure. During anaesthesia, the most prominent adverse effect was respiratory depression. Hypothermia, which generally occurs under anaesthesia, was prevented by placing the animal on a warmed mat. After completion of anaesthesia, altered physiological functions, such as elevated heart rate and core body temperature, persisted for approximately half a day. In conclusion, both isoflurane and sevoflurane provided an equally effective anaesthesia with acceptable adverse effects.

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Combining Sevoflurane Anesthesia with Fentanyl–Midazolam or S-Ketamine in Laboratory Mice

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Laboratory mice typically are anesthetized by either inhalation of volatile anesthetics or injection of drugs. Here we compared the acute and postanesthetic effects of combining both methods with standard inhalant monoanesthesia using sevoflurane in mice. After injection of fentanyl–midazolam or S-ketamine as premedication, a standard 50-min anesthesia was conducted by using sevoflurane. Addition of fentanyl–midazolam (0.04 mg/kg–4 mg/kg) induced sedation, attenuation of aversive behaviors at induction, shortening of the induction phase, and reduced the sevoflurane concentration required by one third (3.3% compared with 5%), compared with S-ketamine (30 mg/kg) premedication or sevoflurane alone. During anesthesia, heart rate and core body temperature were depressed significantly by both premedications but in general remained within normal ranges. In contrast, with or without premedication, substantial respiratory depression was evident, with a marked decline in respiratory rate accompanied by hypoxia, hypercapnia, and acidosis. Arrhythmia, apnea, and occasionally death occurred under S-ketamine–sevoflurane. Postanesthetic telemetric measurements showed unchanged locomotor activity but elevated heart rate and core body temperature at 12 h; these changes were most prominent during sevoflurane monoanesthesia and least pronounced or absent during fentanyl–midazolam–sevoflurane. In conclusion, combining injectable and inhalant anesthetics in mice can be advantageous compared with inhalation monoanesthesia at induction and postanesthetically. However, adverse physiologic side effects during anesthesia can be exacerbated by premedications, requiring careful selection of drugs and dosages.

Abbreviations: FMS, fentanyl–midazolam–sevoflurane; KS, S-ketamine–sevoflurane; S, sevoflurane.

Laboratory mice frequently are anesthetized by subcutaneous or intraperitoneal injection of hypnotic, analgesic, and muscle-relaxing agents.[35] Although easy, practical and cost-effective, this method has its drawbacks. After injection of relatively long-acting drugs through the subcutaneous or intraperitoneal route, the course and depth of anesthesia is nearly uncontrollable once the initial dose has been administered. In addition, due to the considerable variability in dose requirements for mice of different age, strain, sex, and other specifics (for example, circadian rhythm, sociophysiologic conditions), the margin between reaching a state of anesthesia sufficiently deep to provide surgical tolerance and a lethal outcome is usually narrow.[1] Moreover, most injection anesthesia protocols induce a prolonged recovery period accompanied by hypothermia and compromised physiologic function.

Such problems rarely are encountered with inhalation anesthesia, because this method has a short recovery phase and accommodates control of the duration and depth of anesthesia, including expeditious adjustment of the dosage of inhalation anesthetics tailored to the requirements of the individual animal. Therefore, in terms of survival rate, inhalation anesthesia generally is suggested to be safe in mice. However, negative effects on the cardiovascular system combined with depression of respiration are well-known side effects of halogenated volatile anesthetics.[18,19,46] This situation, coupled with the fact that the analgesia provided by monoanesthesia with volatile anesthetics is still controversial,[9,15] justifies a continued search for improvement.

By taking advantage of the well-known synergistic and additive interactions between injectable drugs (analgesics or sedatives) and volatile anesthetics, the dosages of each component can be decreased (relative to its use as a sole agent) while inducing general anesthesia of sufficient depth with fewer side effects.[16,20,32] This approach, sometimes referred to as 'balanced' or 'modular' anesthesia,[49] is used widely in human and veterinary medicine—but only recently has it has begun to be used in mice. Therefore, in the present study, 2 protocols of combined injection and inhalant anesthesia in laboratory mice were established and compared with a standard protocol of inhalant monoanesthesia with a commonly used volatile anesthetic.

Isoflurane and sevoflurane are the 2 of the volatile anesthetics most widely used in human and veterinary anesthesia. We decided to use sevoflurane to provide rapid induction and recovery. Because we considered that volatile anesthetics offer suboptimal analgesia, we focused on injectable agents that could provide sufficient analgesia to complement inhalant anesthesia. Ketamine is known for its ability to cause profound analgesia, which can occur even at subhypnotic dosages—particularly if the S(+)-enantiomer of ketamine is adminstered.[36] Therefore, we chose S-ketamine for injection in one protocol. We calculated the dosage based on literature reports,[17,34,36,37] with the aim of minimizing side effects such as catalepsy, slight respiratory depression, and stimulation of locomotor activity (restlessness) while inducing analgesia and taking advantage of the hypnotic

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and cardiovascular stimulatory effects of ketamine.[17,30] Our second approach to combining inhalation anesthesia with injectable agents in mice was based on drugs that are used widely in human medicine, namely fentanyl and midazolam. Midazolam, which often is applied as premedication to anesthesia, belongs to the benzodiazepines, which typically induce sedation, anxiolysis, and muscle relaxation.[28] Antinociceptive effects of midazolam have been reported in mice[8] and rats.[5] In humans, benzodiazepines frequently are administered with opioids to improve pain relief. Therefore, we combined midazolam with fentanyl—a potent synthetic opioid analgesic. Among the typical side effects of opioids,[26] sedation, hypothermia, respiratory depression, and hypercapnia could be of relevance for the use of fentanyl during anesthesia in mice. Although opioids can cause bradycardia, vasodilation, and hypotension, they have mostly only mild effects on cardiovascular function. In addition, their effects on the genitourinary system and gastrointestinal tract[26] (for example, constipation) are suggested to be tolerable side effects, which may be of only minor relevance for establishing an anesthesia protocol in mice. Fentanyl often is administered as an intravenous constant-rate infusion in the context of anesthesia in humans,[48] but this technique is complex and difficult to manage in mice. Therefore, we attempted to achieve preemptive analgesia with subcutaneous injection of fentanyl with midazolam as premedication, with dosages selected on the basis of anecdotal evidence, clinical experience, and hints from the literature.[47]

To compare the 3 anesthesia protocols, 50 min of sevoflurane inhalant anesthesia was conducted either alone (S) or with subcutaneous injection of S-ketamine (KS) or a mixture of fentanyl and midazolam (FMS). Injections were administered as premedication, and their effects on behavior during induction of anesthesia and on the sevoflurane concentration required were noted. During anesthesia, heart rate, core body temperature, respiratory rate, arterial blood gases, and arterial pH were monitored. The long-term effect of the 3 protocols on recovery from anesthesia was investigated through telemetric measurements of heart rate, core body temperature, and locomotor activity for 3 d after anesthesia.

Materials and Methods

Animals and housing conditions. Female C57BL/6J mice (n = 98; age, 6 wk) were obtained from our inhouse breeding colony. The 72 mice used for determination of minimal alveolar concentrations were later euthanized to obtain arterial blood for measuring acid–base balance and blood gas concentrations. The remaining 26 mice were implanted with telemetric transmitters prior to the experiments to allow measurement of heart rate, core body temperature, and locomotor activity. The mice were free of all viral, bacterial, and parasitic pathogens listed in the FELASA recommendations.[33] Health status was monitored by a sentinel program throughout the experiments.

Mice generally were housed in pairs; each transmitter-implanted mouse was housed with a nonimplanted companion of the same strain, sex, and age. Mice were kept in Eurostandard type III open-top plastic cages (425 mm × 266 mm × 155 mm, floor area 820 cm^2, Tecniplast, Indulab, Gams, Switzerland) with autoclaved aspen bedding (80 to 90 g per cage; LTE E-001 Abedd, Indulab). Autoclaved hay (8 to 12 g per cage) and 2 cotton nesting pads (each 5 × 5 cm; Nestlets, Indulab) were provided as nesting materials. A standard cardboard house (Ketchum Manufacturing, Brockville, Canada) served as a shelter. Mice were fed a pelleted mouse diet (3431, Provimi Kliba, Kaiseraugst, Switzerland) ad libitum and had unrestricted access to sterilized drinking water provided in a water bottle. The 12:12-h light:dark cycle in the room was established with artificial light (approximately 40 lx in the cage; lights on, 0300 to 1500). The temperature was 21 ± 1 °C, with a relative humidity of 50% ± 5% and 15 complete changes of HEPA-filtered air hourly. To avoid interfering influences, all necessary husbandry and management procedures were completed in the room at least a d before the start of an experiment or data recording, and disturbances (for example, visitors or unrelated experimental procedures) were not allowed. The animal room was insulated to exclude electronic noise.

The study and all procedures and protocols were approved by the Cantonal Veterinary Office (Zurich, Switzerland) under license number 111/2007. Housing and experimental procedures were in accordance with Swiss animal protection law and conformed to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Council of Europe nr.123 Strasbourg 1985).[10] Housing and experimental procedures also were in accordance with the *Guide for the Care and Use of Laboratory Animals*[22] and conformed to the AALAS position statement on the humane care and use of laboratory animals.

Transmitter implantation. Prior to the experiments, at age 10 wk, 26 mice were instrumented with telemetric transmitters (TA10ETA-F20, Data Sciences International, St Paul, MN) to measure heart rate, core body temperature, and locomotor activity in freely moving mice.[2,44] Briefly, mice were anesthetized with sevoflurane (Sevorane, Abbott, Baar, Switzerland), and the transmitter body was implanted in the abdomen under aseptic conditions. One wire-loop electrode was fixed with silk sutures (6-0 Perma-Handseide, Ethicon, Norderstedt, Germany) between the muscles located to the right of the trachea, and the other loop lead was sutured to the xiphoid process. Muscle layers and skin were closed with resorbable sutures (6-0 Vicryl, Ethicon). Postoperative pain was treated with flunixine (5 mg/kg SC twice daily; Biokema Flunixine, Biokema SA, Crissier-Lausanne, Switzerland) for 4 d.[38] After transmitter implantation, mice were allowed to recover for 6 wk before the first experiment.

Experimental setting. All experiments were conducted when the mice were 16 to 36 wk of age, with body weights ranging from 25 to 30 g. To avoid any influence of circadian rhythm, all experiments and weighing procedures were done between 1500 and 1800. The study was designed for the experiments and anesthesia to be performed at the beginning of the dark phase for these mice. Anesthesia was performed in a separate operating area within the animal room to avoid transportation of the mice and to ensure stable conditions of humidity, air pressure, and room temperature and sufficient removal of gases and smells through the ventilation system.

The method of delivering inhalation anesthesia was modified slightly from that described elsewhere.[6] Briefly, sevoflurane was provided by using a commercially available rodent inhalation anesthesia apparatus (Provet, Lyssach, Switzerland), which was equipped with a sevoflurane vaporizer (Ohmeda Sevotec 5, Abbott, Baar, Switzerland) and a pump-driven filter system to eliminate waste anesthetic gas. As carrier gas, pressurized air was used at a flow rate of 600 mL/min. The anesthetic gas was introduced into the induction chamber or nose mask (Figure 1).

Premedications. As injectable drugs, we used fentanyl (0.04 mg/kg SC; Kantonsapotheke Zurich, Zurich, Switzerland) mixed with midazolam (4 mg/kg SC; Dormicum, Roche Pharma Schweiz AG, Reinach, Switzerland) in one protocol and S-ketamine (30 mg/kg SC; Keta-S, Graeub AG, Bern, Switzerland)

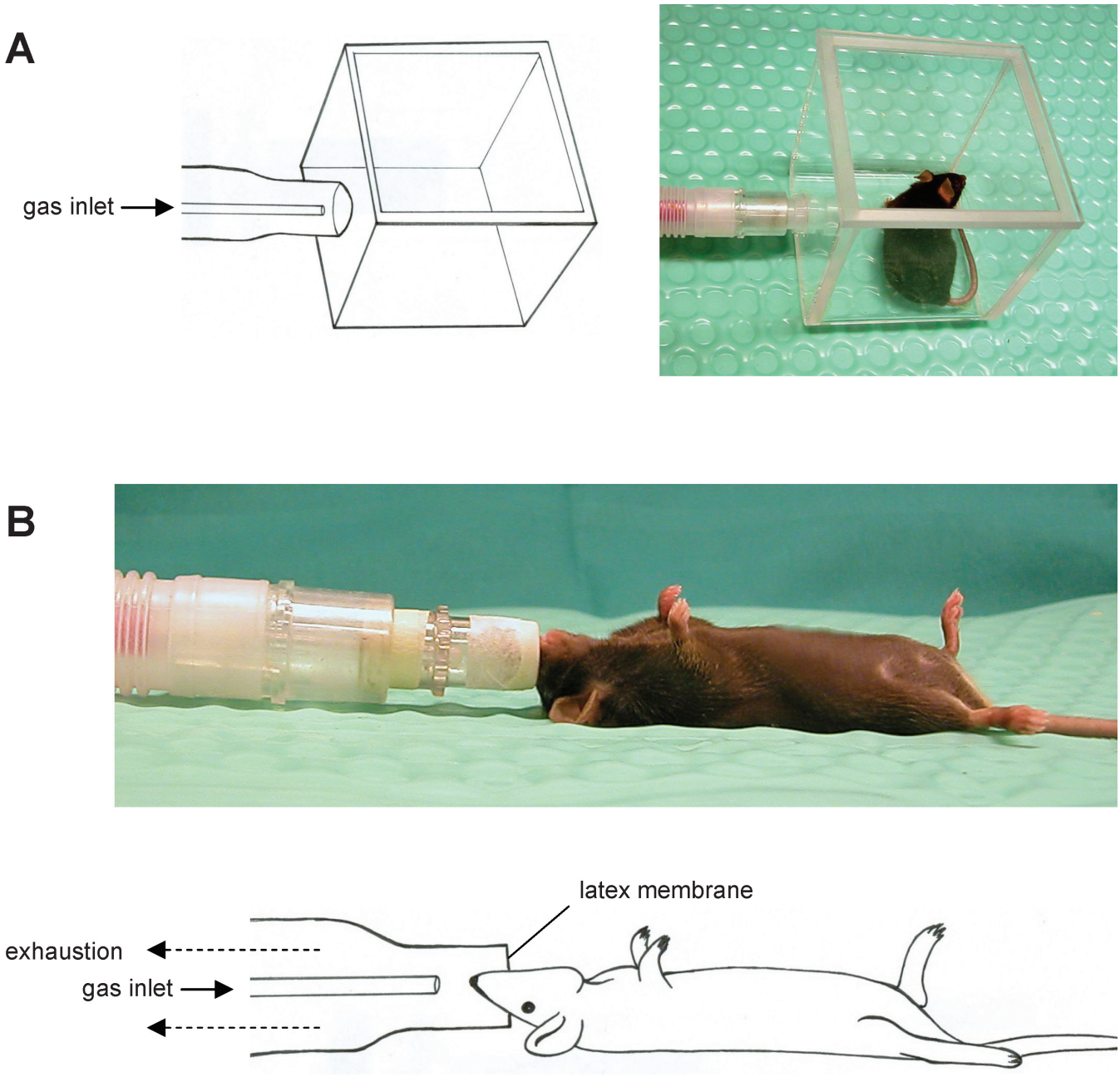

Figure 1. (A) Chamber for induction of sevoflurane inhalation anesthesia. (B) Nose mask for maintaining sevoflurane anesthesia. The mask was equipped with a latex membrane, which had a hole in the center that fit around the nose of each mouse, with the dual purpose of preventing both withdrawal of environmental air into the nose mask and leakage of anesthetic gas from it.

in another. All drugs were dissolved in PBS immediately before injection in such a manner that dosing could be achieved by application of an injection volume of 2 µL/kg body weight. Injections were provided as premedication, that is, 5 to 7 min before sevoflurane anesthesia was induced.

Determination of minimal alveolar concentration. Sevoflurane inhalation anesthesia was standardized by establishing minimum alveolar concentrations during sevoflurane monoanesthesia and after the premedications described earlier. To this end, we anesthetized 72 nontransmitter-implanted mice 2 to 4 times each; care was taken that mice had a break of at least 2 wk between tests.

Minimal alveolar concentration was determined according to commonly accepted procedures used in mice.[6,7,14,23,42,43] For each protocol, 4 consecutive sevoflurane concentrations differing by 0.25% were tested; 25 mice were tested per concentration. The bracketed study design[41] was adapted to our anesthesia protocols to measure minimal alveolar concentration at a defined time point of anesthesia, that is, at 12 min after inducing inhalant anesthesia (equivalent to 17 to 19 min after subcutaneous injection of premedication). Thus, after inducing sevoflurane anesthesia in the induction chamber (Figure 1 A) for 1.5 min at a maximal concentration of 8% sevoflurane, the mouse was taken out of the chamber and placed in dorsal recumbency on a warmed mat. Anesthetic gas then was applied at the test concentration by using a nose mask, with the mouse breathing spontaneously (Figure 1 B). After an equilibration time of 10 min, 3 noxious stimuli were applied sequentially: pinching of the tail (tail pinch reflex), interdigital webbing (pedal withdrawal reflex), and abdominal skin (abdominal skin pinch reflex). All stimuli were induced by the same investigator by using

blunt forceps with a spacer between its arms to allow uniform application of pressure. Any motor response (for example, movement of the tail or an extremity, head jerking) to one or more of the 3 noxious stimuli was judged as purposeful movement, indicating that sevoflurane at the concentration applied did not induce anesthesia in the mouse evaluated. After testing the response to the 3 noxious stimuli (that is, after 12 to 13 min of inhalant anesthesia), administration of the anesthetic gas ceased, and the mouse was allowed to recover. By using the responses to the noxious stimuli, the mouse's minimal alveolar concentration was calculated as the average of the 2 partial pressures bracketing the positive response (that is, purposeful movement) or lack of response in the animal.

Anesthesia experiments. Mice were allocated randomly to 1 of 3 anesthesia protocols. The 3 protocols consisted of fentanyl (0.04 mg/kg) and midazolam (4 mg/kg) as premedication and 3.3% sevoflurane (FMS); S-ketamine (30 mg/kg) as premedication and 5% sevoflurane (KS); and 5% sevoflurane as monoanesthesia (S). After premedication in the FMS and KS protocols, the mice were examined for 5 to 7 min in their home cage for behavioral aberrations. Inhalant anesthesia then was induced by placing each mouse in a clear induction chamber (8 × 8 × 8 cm; volume, 512 mL) into which 8% sevoflurane (Sevorane, Abbott, Baar, Switzerland) was introduced. The mouse's behavior in the induction chamber and the time point at which it became immobile were observed and noted. After 1.5 min, the mouse was transferred rapidly to the nose mask, through which anesthesia was maintained with sevoflurane. Mice breathed spontaneously while lying in dorsal recumbency on a water-filled warming mat (Gaymar, TP500, Orchard Park, NY) set at 39 °C ± 1 °C.

Tail pinch, pedal withdrawal, and abdominal skin pinch reflexes each were tested at 5-min intervals. All reflex tests were induced by the same investigator by using blunt forceps with a spacer between its arms to allow uniform application of pressure. The reflex tests were registered as positive or negative (that is, whether any motor response was present or not).

Respiratory rate was counted from the movement of the thorax wall and recorded at 5-min intervals. During anesthesia, mice were observed for any abnormality in their respiratory rhythm. In addition, heart rhythm alterations were monitored by using real-time telemetric electrocardiograms.

Anesthesia was stopped after 50 min by removing the nose from the mask and letting the mouse breathe room air. Mice were left on the warming mat and allowed to recover from anesthesia for 10 min before being placed back in their home cages.

Telemetric data acquisition and analysis. Telemetric data were recorded from 8 mice per anesthetic protocol by using the Dataquest LabPRO program (Data Sciences International). Data collection was initiated by switching on the transmitter by using a magnet. Data acquisition started 3 d before anesthesia and continued for 3 d after anesthesia.

To estimate the acute effects of anesthesia (that is, after premedication and during anesthesia), heart rate and core body temperature were recorded for 4 s every 15 s (4 data points of 4 s per minute). From these data, mean values of heart rate and core body temperature were calculated for each minute for each mouse. Baseline values represent means from 1500 to 1800 (that is, the same time frame during which anesthesia occurred) during the 3 d prior to the experiment.

To establish baseline values (3 d before anesthesia) and to investigate postanesthetic effects (3 d after anesthesia), heart rate was measured for 30 s every 5 min, and core body temperature was measured for 10 s every 5 min. Locomotor activity was recorded continuously and stored at 5-min intervals.

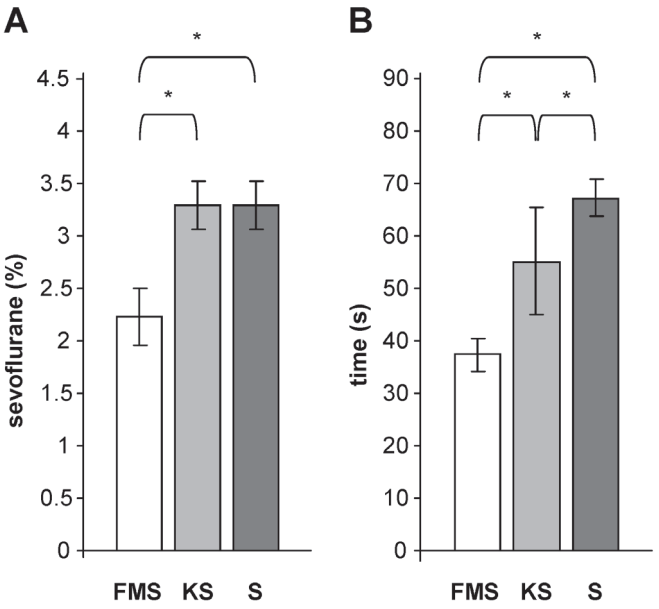

Figure 2. (A) Mean (n = 50 mice; bar, 1 SD) minimum alveolar concentrations for sevoflurane in adult C57BL/6J female mice. The gas-saving effect is evident from the decrease in minimum alveolar concentration seen after fentanyl–midazolam premedication with sevoflurane (FMS) compared with S-ketamine premedication with sevoflurane (KS) and sevoflurane alone (S). *, $P \leq 0.05$ between values. (B) The mean time (n = 8 mice; bar, 1 SD) required until immobilization after mice were placed in the sevoflurane-filled induction chamber differed between all protocols. *, $P \leq 0.05$ between values.

For analysis of long-term postanesthetic effects, we took into account that values vary greatly during a 24-h cycle because mice are active mainly at night. Therefore, the means of the telemetric values for each mouse were calculated separately for the 12-h dark (night) and 12-h light (day) phases. A mouse's baseline values were established by calculating means from the 3 d prior to anesthesia. For each day after anesthesia, a mouse's baseline value was subtracted individually from its daytime and nighttime means; the differences are reported as delta (Δ) values.

Changes in body weight. Body weight in transmitter-implanted mice was monitored for 3 d before and 3 d after anesthesia. Weights were obtained by using a precision balance (PR 2003 Delta Range, Mettler-Toledo AG, Greifensee, Switzerland) that specifically was adjusted for use with moving animals. Body weights were corrected to account for the weight of the transmitter (3.6 g). Mean baseline weight (from 3 consecutive daily measurements prior to the experiment) was calculated for each mouse and compared with that recorded on each of the 3 d after the experiment.

Acid–base balance and blood gas concentration. At 3 to 4 wk after determination of minimal alveolar concentration determination, arterial blood was collected from the same 72 nonimplanted mice to assess acute effects of anesthesia on respiration and acid–base balance. Arterial blood was obtained after 10, 30, and 50 min of anesthesia from 8 mice per anesthetic protocol and time point.

Blood sampling and analyses were carried out as described previously.[1,6] Briefly, the anterior neck was incised, the right common carotid artery was dissected out, a small hole in the artery was created by using fine-blade scissors, and arterial blood was collected in a heparinized syringe. Acid–base balance (pH), pCO_2 (mm Hg), and pO_2 (mm Hg) were determined immediately by using a blood-gas analyzer (Compact 3, AVL List, Graz, Austria). These mice died immediately due to the

Table 1. Behaviors of mice (n = 8 per protocol) during induction of anesthesia with sevoflurane.

	% of animals showing					
Anesthesia	Locomotion with or without ataxia	Jumping	Shaking head or limbs or both	Urination	Defecation	Apnea or death
FMS	12.5	0	0	0	0	0
KS	100	0	0	37.5	0	12.5
S	100	50	100	100	62.5	0

subsequent rapid loss of blood under anesthesia. Reference values of pH, pCO_2, and pO_2 for comparison had been established by using arterial blood from 20 HanIbm:NMRI mice that were similar in age to those in the current study.[1]

Statistical analysis. All data are presented as mean ± 1 SD. Statistical analysis (version 17.0, SPSS for Windows, SPSS, Chicago, IL) was done to validate the results. All data were tested for normal distribution and homogeneity of variance and met the necessary assumptions for parametric analyses. One-way ANOVA was performed to compare group means of minimal alveolar concentrations and time until immobilization as well as heart rate, core body temperature, and locomotor activity at each of the first 3 d after anesthesia. Post hoc analysis with Bonferroni tests was done to identify significant differences between groups. For comparison of baseline values with corresponding experimental group means of heart rate, core body temperature, and locomotor activity during and at each of the 3 d after anesthesia, a dependent t test for paired samples was used. P values less than or equal to 0.05 were considered significant.

Results

Minimal alveolar concentration. The minimal alveolar concentration (mean ± 1 SD) for sevoflurane monoanesthesia and with premedication using S-ketamine in adult female C57BL/6J mice was 3.3% ± 0.18% (Figure 2 A). Premedication with fentanyl–midazolam significantly (P = 0.0005) decreased the mean minimal alveolar concentration for sevoflurane to 2.2% ± 0.27% compared with that for the other 2 protocols. This decrease represents a gas savings of 33%.

We considered that providing sevoflurane at 1.5 times the minimal alveolar concentration would prevent mice from responding to noxious stimulation (that is, surgical tolerance is achieved). Therefore all subsequent anesthesia experiments were conducted by using 3.3% sevoflurane after fentanyl–midazolam premedication but by using 5% sevoflurane after S-ketamine premedication and during sevoflurane monoanesthesia.

Induction of anesthesia. Approximately 2 min (107.5 ±18.3 s) after injection with fentanyl–midazolam, all mice showed signs of sedation (for example, absence of locomotion and stationary activity, sleep-like posture). Approximately 5 min (306 ± 55.8 s) after injection with S-ketamine, all mice exhibited symptoms of tremor, ataxia, and dizziness.

When placed in the induction chamber, most nonpremedicated mice (that is, the sevoflurane monoanesthesia group) showed behaviors including defecation, urinating, shaking the head or limbs, jumping, and locomotion (Table 1). These behaviors were less frequent after S-ketamine premedication and were nearly totally absent after fentanyl–midazolam premedication. One transmitter-implanted mouse died after S-ketamine premedication when the animal was exposed to sevoflurane in the induction chamber; this animal was replaced.

The time until immobilization differed among all 3 protocols. The shortest time was associated with the FMS protocol and the longest with sevoflurane monoanesthesia (FMS compared with S, P = 0.0005; FMS compared with KS, P = 0.0005; KS compared with S, P = 0.004; Figure 2 B).

Effects during anesthesia. During anesthesia, none of the mice showed any motor response to testing of the pedal withdrawal reflex, tail pinch, or abdominal skin pinch.

During the 50-min anesthesia period in all 3 protocols, heart rate and core body temperature remained within the general physiologic boundaries for this species (350 to 800 bpm, 35 to 38 °C; Figure 3). Heart rate was 446 ± 51 bpm during FMS anesthesia, 470 ± 59 bpm during KS anesthesia, and 519 ± 60 bpm during sevoflurane monoanesthesia. Compared with the mean baseline heart rate at the corresponding time of day (525 ± 80 bpm), the decreases in heart rate during FMS (P = 0.001) and KS (P = 0.030) were significant.

Compared with the baseline core body temperature at the same time of day (36.8 ± 0.7 °C), core body temperature was decreased significantly during FMS (35.4 ± 0.6 °C; P = 0.0005) and KS (35.4 ± 0.4 °C; P = 0.0005) anesthesia. Core body temperature showed a tread toward a decrease during sevoflurane monoanesthesia (36.1 ± 0.7 °C; P = 0.058).

During all 3 protocols, the respiratory rate declined immediately after the onset of sevoflurane anesthesia and remained markedly depressed during the 50-min anesthesia session compared with baseline respiration in resting mice at the same time of day (150 ± 10 breaths per minute; Figure 3). The respiratory rate during anesthesia was 68.5 ± 7.7 breaths per minute for FMS, 48.8 ± 5.4 breaths per minute for KS, and 44 ± 5.1 breaths per minute for sevoflurane monoanesthesia. After 10, 30 and 50 min of anesthesia in all 3 protocols, blood gas and pH measurements of arterial blood showed prominent acidosis, hypercapnia, and hypoxia, with values markedly exceeding the physiologic range (Figure 4).

During anesthesia with KS, all mice displayed cardiac arrhythmia and episodes of apnea followed by tachypnea. None of these events occurred during either of the other 2 protocols. One transmitter-implanted mouse died at 15 min into KS anesthesia and was replaced.

Mice began showing increasing respiratory rate and muscle rigor within 1 min after sevoflurane was discontinued. In all 3 protocols, the mice had turned to ventral recumbency and were able to move within approximately 2 min after sevoflurane withdrawal.

Effects during the first 3 d after anesthesia. Compared with baseline values, telemetric measurements revealed a significant (P = 0.0005) increase in heart rate during the first 12 h after anesthesia in all 3 protocols (Figure 5). Comparing between protocols, the increase in heart rate after sevoflurane monoanesthesia was significantly (P = 0.0005) higher than that after FMS anesthesia, whereas heart rate after KS anesthesia did not differ significantly from that in the other 2 protocols.

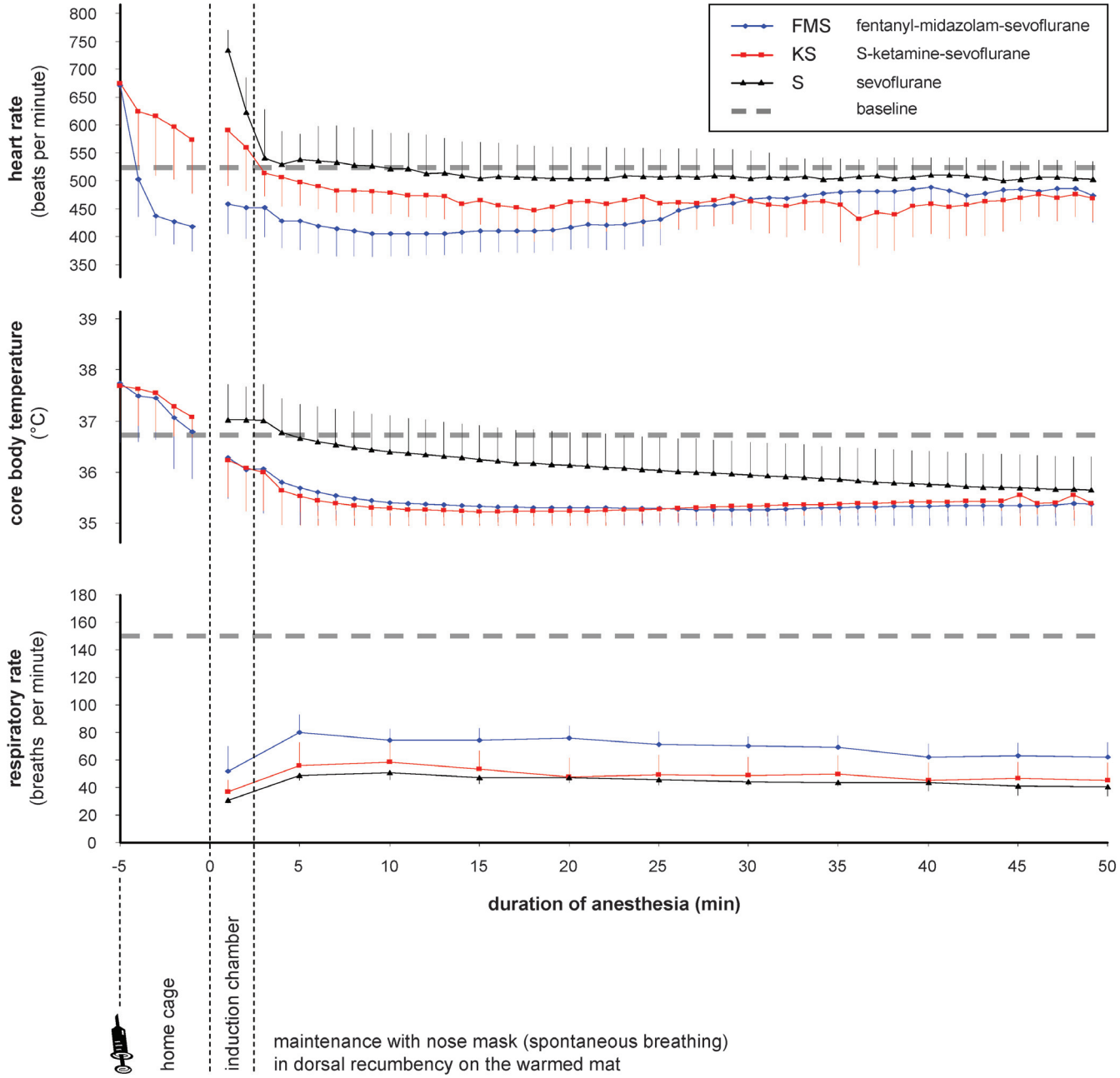

Figure 3. Mean (n = 8 mice; bar, 1 SD) heart rate, core body temperature, and respiratory rate after premedication in the home cage, in the induction chamber, and during 50-min sevoflurane anesthesia while mice breathed spontaneously and lay in dorsal recumbency on the warming mat. Dashed lines indicate mean baseline values (measured before anesthesia) at the same time of day in conscious mice. The baseline respiratory rate was established by counting the movement of the thorax wall in resting mice before anesthesia.

Compared with baseline values, core body temperature increased significantly during the first 12 h after anesthesia with KS anesthesia (P = 0.005) and sevoflurane monoanesthesia (P = 0.0005) but not after FMS anesthesia (Figure 5). Core body temperature was significantly higher after sevoflurane monoanesthesia compared with KS and FMS (S compared with FMS, P = 0.0005; S compared with KS, P = 0.006).

Locomotor activity and body weight were unchanged in all groups after anesthesia relative to baseline values before anesthesia.

Discussion

All 3 protocols tested provided a reliable 50-min period of anesthesia in laboratory mice, with short induction and recovery phases and lack of motor response to noxious stimuli. Subcutaneous injection of fentanyl–midazolam prior to sevoflurane inhalant anesthesia induced a gas-saving effect and had the advantage of inducing immediate sedation and preventing aversive reactions as well as extensive movements at the time of induction with sevoflurane. Injection of S-ketamine, the S(+)-enantiomer of ketamine, initially induced behavioral aberrations suggestive of excitation but attenuated aversive behaviors when mice were exposed to sevoflurane. In contrast, when sevoflurane anesthesia was induced without premedication, mice responded with defecation, urination, and locomotion including jumping and abnormal stationary movements. Compared with sevoflurane monoanesthesia, both premedication regimens shortened

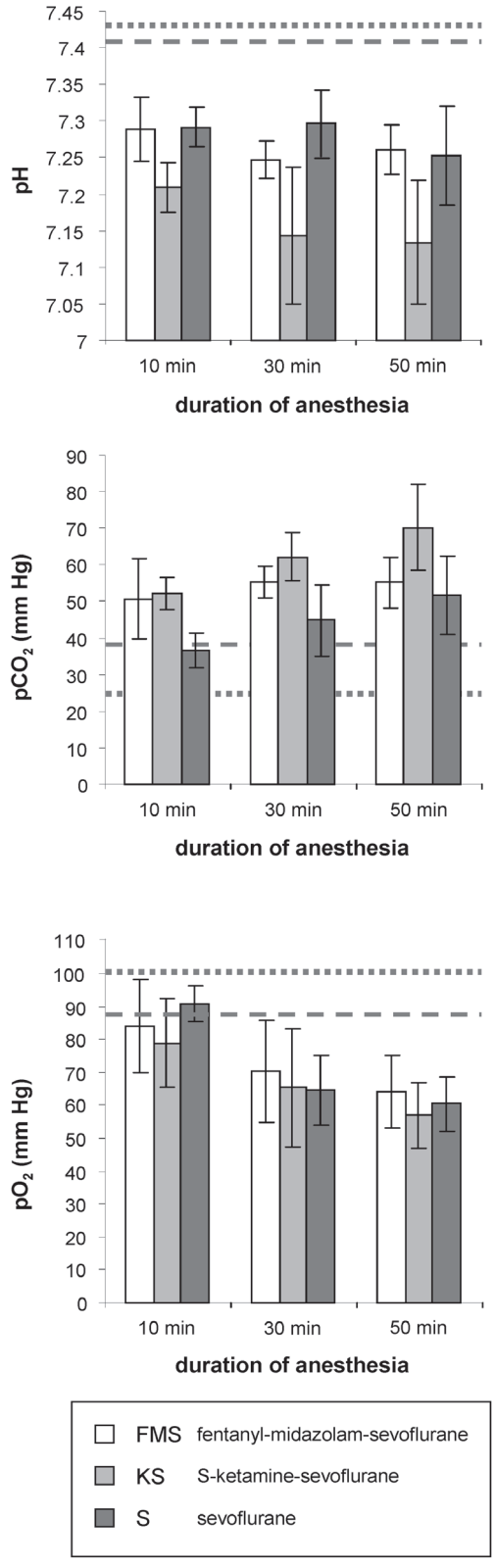

Figure 4. Mean (n = 8 mice; bar, 1 SD) acid–base balance (pH), pCO_2, and pO_2 in arterial blood after 10, 30, and 50 min of sevoflurane anesthesia. Dotted lines indicate baseline levels established from HanIbm:NMRI mice in a previous study.[1] Dashed lines indicate published values from conscious C57BL/6J mice.[24]

the time required to reach immobilization after exposure to sevoflurane; this effect was most pronounced with fentanyl–midazolam.

During anesthesia, while mice were warmed by a water-filled mat, core body temperature and heart rate were depressed compared with baseline values obtained at the time of day but before anesthesia. Both premedications intensified these effects, but all values during all 3 protocols remained within the ranges considered to be normal for mice. The most important adverse side effect that occurred during anesthesia was marked respiratory depression, as indicated by respiratory rates that were far below those of normal resting mice. This respiratory depression was accompanied by pronounced hypoxia, hypercapnia, and acidosis, all of which increased with time during anesthesia. Such changes in acid–base balance and blood gasses are well-known side effects of inhalant as well as injectable anesthesia.[3,6,46] The degree of respiratory depression was nearly equal among all protocols, but apnea, tachypnea, and cardiac arrhythmia occurred with KS anesthesia, and 2 mice in this group died.

During the first 12 h after anesthesia, heart rate increased in all protocols; this increase was most pronounced during sevoflurane monoanesthesia and least apparent during the FMS protocol. Core body temperature was increased at 12 h after sevoflurane monoanesthesia and to a lesser extent after KS anesthesia. Because locomotor activity was unchanged after anesthesia regardless of protocol, physical activity is unlikely to be the reason for these effects. Postanesthetic measurements, including monitoring of body weight, indicated that all 3 protocols had only a short-term effect on the physiology and general condition of the mice.

The minimal alveolar concentration of sevoflurane was determined according to standard principles,[41] including generally accepted adaptations for the particular species-specific conditions of mice.[23,42,43] These modifications mainly concern the fact that constant-rate infusions and mechanical ventilation are not performed during determination of minimal alveolar concentration in mice. Furthermore, measurements of minimal alveolar concentration in mice were based on the inspired concentration of the inhalant, instead of on the end-tidal value, as is typical for larger animal species. In addition to the common single noxious stimulus induced by pinching the tail of the mouse,[7] we applied 2 other noxious stimuli. The hindlimb withdrawal reflex has been shown to be useful for estimating depth of anesthesia in mice.[24] Because applying a clamp between the toes was described as useful during the determination of minimal alveolar concentration of isoflurane in mice,[14] we incorporated this stimulus in the form of pinching the interdigital webbing of the paw (pedal withdrawal reflex) in a reproducible manner. As a third noxious stimulus, the abdominal skin pinch reflex was applied as described earlier.[1] For determination of the minimal alveolar concentration of sevoflurane, we applied these 3 noxious stimuli only once at a predefined time point of inhalant anesthesia to standardize the experimental conditions in regard to sevoflurane concentration and the single injection of fentanyl–midazolam or S-ketamine, with a view to determining the pharmacokinetics of the injected agents. Therefore, minimal alveolar concentration was determined at 12 min of sevoflurane anesthesia, which is congruent with 17 to 19 min after subcutaneous injection of the premedication.

The minimal alveolar concentration determined for sevoflurane monoanesthesia (3.3%) for the female C57BL/6J mice we tested here was similar to values in from the literature.[6,29] Analgesic substances are known to reduce the minimal alveo-

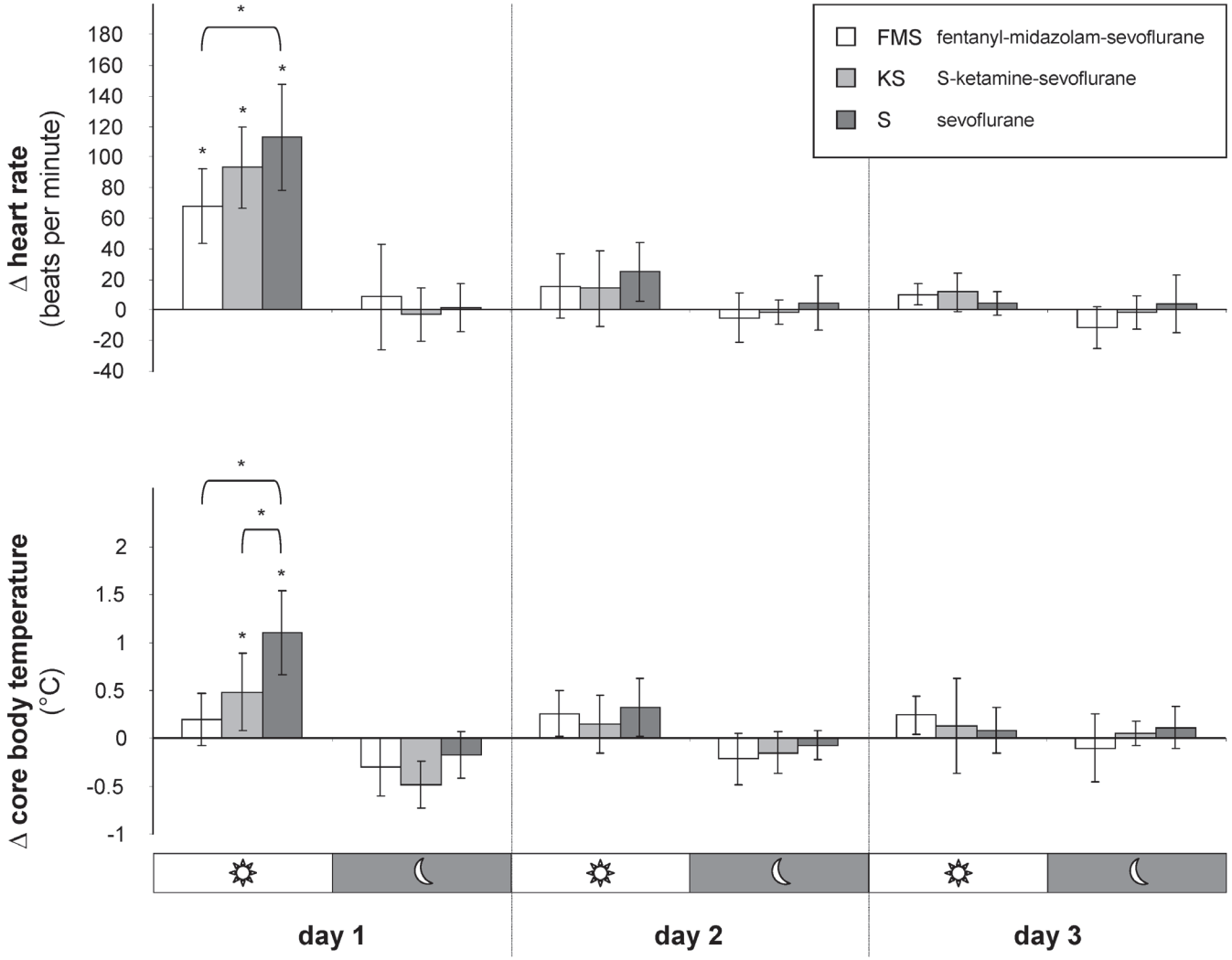

Figure 5. Mean (n = 8 mice; bar, 1 SD) postanesthetic measurements of the effects of 3 anesthesia protocols on heart rate and core body temperature. Delta (Δ) values represent deviations from baseline values (established prior to anesthesia) during the corresponding 12-h day and night periods. *, $P \leq 0.05$ compared with baseline values and between protocols.

lar concentration during inhalant anesthesia in many animal species.[12,51] In humans, both fentanyl and midazolam induce a gas-saving effect when combined with volatile anesthetics.[21,31,39] In the current study, applying 0.04 mg/kg fentanyl in combination with 4 mg/kg midazolam as a subcutaneous bolus injection prior to anesthesia reduced the requirement for sevoflurane gas by one third. A similar gas-saving effect with isoflurane has been described for ketamine in dogs,[40] but combination of S-ketamine with sevoflurane did not have this effect in our mice. The most probable explanation for this lack is that we could not administer S-ketamine as a target-controlled intravenous infusion (as is possible in large animals and humans) but rather as a single subcutaneous bolus injection. Therefore, from a pharmacokinetic viewpoint, the effects of S-ketamine might already have been decreasing when we determined the minimal alveolar concentration (that is, at 17 to 19 min after subcutaneous injection of 30 mg/kg S-ketamine).[36]

After standardization of the dosages by establishing minimal alveolar concentrations, we then compared the 3 protocols at dosages of sevoflurane representing 1.5 times the minimal alveolar concentrations. At this dosage, it is generally postulated that 99.9% of animals will not react to noxious stimuli,[11,13] that is, that the animals have reached surgical tolerance. However, because we did not confirm analgesia by, for example, measuring heart rate, blood pressure, or respiration in response to a substantially noxious stimulus (for example, skin incision), we cannot claim definitively that surgical tolerance was achieved in the current study. However, motor reflex responses to noxious stimuli were suppressed in all mice for the entire duration of anesthesia (that is, 50 min).

Shortly (within approximately 2 min) after injection with fentanyl–midazolam, all mice exhibited reduced physical activity and a sleep-like posture, likely due to the sedative effect of these agents. In contrast, injection of S-ketamine gave rise to muscle tremors and ataxia. The spike (up to 800 bpm) in heart rate that we noted in the early phase of induction during sevoflurane monoanesthesia may be a normal reaction to removal of the mouse from its cage and placing it in a foreign environment (that is, induction chamber). The markedly lower heart rate during the induction phase of the FMS protocol suggests bradycardia due to fentanyl but also indicates the potential benefits of sedation, through stress reduction, during the initial phase of anesthesia.

During the 50-min anesthesia, mice anesthetized with FMS and KS displayed lower heart rate and core body temperature than did those anesthetized with S alone. This result can be explained by the known influences of fentanyl and ketamine

on thermoregulation.[4,50] In addition, the typical cardiovascular effects of the opioid might have potentiated the well-known cardiopulmonary depression caused by the volatile anesthetic sevoflurane. However, all heart rate and core body temperature measurements remained within the normal physiologic ranges of mice in all 3 protocols tested. Given that many other anesthetic regimens can decrease in core body temperature by more than 4 °C in just a few minutes, we consider the changes in the current study to be acceptable.[1,52] Nevertheless, these findings underline the necessity for thermal support (as supplied in our experiments) during anesthetic procedures in small laboratory rodents.

In all protocols, the respiratory rate declined far below baseline values in resting mice. Blood gasses and pH in the arterial blood were impaired by all protocols to a similar extent and to values clearly different from published reference values from conscious HanIbm:NMRI and C57BL/6J mice.[1,27] Therefore, respiratory depression—as evidenced by the marked decrease of respiratory rate in the presence of acidosis, hypoxia, and hypercapnia—was the most prominent side effect observed and it was present in all of the anesthetic protocols we tested. These symptoms may mainly reflect the effect of sevoflurane on cardiopulmonary function but also may be potentiated by fentanyl and—to a lesser extent—through S-ketamine. Although surgical stimulation can restore ventilation toward a less deleterious level,[25,45] surgery was not performed in our experiments. Therefore, lack of constant surgical stimulation may have exacerbated the respiratory depression associated with duration of anesthesia. The oxygen content of the air used as a carrier gas likely is insufficient to prevent hypoxia. Therefore, increasing the inspiratory oxygen fraction (FiO_2) above 0.3 by mixing oxygen into the carrier gas would be useful to minimize hypoxia due to anesthetic-induced respiratory depression.

The time required for recovery (that is, resposture and motion) after anesthesia was similar for all groups (1 to 2 min). This fact should be highlighted as a distinct advantage over most injectable anesthetic regimens in mice.[1] The avoidance of postoperative complications associated with a prolonged recovery period and the resulting inability to correct physiologic impairment (for example, hypoxia, hypothermia, hypoglycemia, dehydration) is a key challenge in developing novel anesthesia methods for small laboratory rodents.

Heart rate and core body temperature increased in all 3 protocols during the first 12 h after the 50-min anesthesia, but these changes were attenuated or absent in mice that received FMS anesthesia. Therefore, the influence of our tested anesthesia protocols on physiology seemed to be of only short-term duration. It should also be noted that, during the 3 d after anesthesia, none of the anesthetic protocols showed an adverse effect on body weight, thus suggesting their negligible effects on the animals' general condition.

In the current study, we tried to apply the most useful and efficient substances and dosages of injectable drugs and volatile anesthetic available for the adult female C57BL/6J mice we used. Because dosages vary greatly depending on the specific characteristics of the animals used (for example, strain, age, sex) as well as the ambient conditions of the laboratory, our protocols should carefully be adapted for use in other circumstances. Depending on the animals' anesthetic needs and the severity of the intervention that they will undergo, higher or lower dosages—particularly of the volatile anesthetic sevoflurane—may be required. In addition, we suggest that the use of S-ketamine as a premedication might be improved by administering it at a lower dose or in combination with a minor tranquilizer. Such optimization could decrease the frequency, duration, and severity of side effects such as apnea, arrhythmia (including fatal abnormalities), and excessive excitation.

In conclusion, premedication with subcutaneous injection of fentanyl in combination with midazolam improved standard sevoflurane monoanesthesia of mice in our laboratory setting. Advantages included a short and quiet induction phase and decreased negative postanesthetic side effects on heart rate and core body temperature. A gas-saving effect was evident in the FMS treatment, corroborating the analgesic potential of the opioid component (fentanyl) in this modular anesthesia protocol.

Although all 3 protocols used here may be useful for anesthesia in mice, the combination of injection anesthesia with inhalation anesthesia could be superior to the widely used standard inhalation monoanesthesia, provided that appropriate drugs are combined and dosages are adapted to the requirements of the specific animals and laboratory. However, the choice of a specific anesthetic regimen should always be based on careful deliberation, considering arguments of animal welfare, feasibility, and any potential interference with the research project for which the anesthesia is required.

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Publications, Abstracts, Invited Talks & Grants:

Published Articles

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Wueest S, Boyle C, Rytka J, Jirkof P, Cesarovic N, Ellingsgaard H, Donath M, Arras M, Lutz T, Schoenle E, Konrad D (2010) Interleukin-6 as a key mediator of metabolic adaptation to fasting. SGED, Bern, 2010

Jirkof P, Cesarovic N, Rettich A, Arras M, (2010) Refinement: analgesia and clinical signs in the framework of product development. EPAA Conference, Brüssel 2010

Jirkof P, Leucht K, Cesarovic N, Hausmann M, Arras M (2011) Monitoring of general condition and animal wellbeing in a murine model of DSS induced colitis. 11th Day of Clinical Research, Zurich, 2011

Jirkof P, Cesarovic N, Leucht K, Rettich A, Hausmann M, Arras M (2011) Burrowing behavior for monitoring of murine colitis. Wissenschaftliche Tagung der Gesellschaft für Versuchstierkunde GV-SOLAS, Dresden, 2011

Cesarovic N, Jirkof P, Arras M (2011) Post-operative change of home-cage behaviour profile in laboratory mice. Wissenschaftliche Tagung der Gesellschaft für Versuchstierkunde GV-SOLAS, Dresden 2011

Jirkof P, Cesarovic N, Rettich A, Nicholls F, Seifert B, Arras M (2011) Assessment of post-surgical pain in mice using species-typical burrowing behaviour. 8th World Congress on Alternatives and Animal Use in the Life Sciences, Montreal, 2011 (**Poster award** Theme IV: Animal Welfare for Refinement and High Quality Science)

"Pain assessment in mice using species-typical burrowing behaviour" 49.Wissenschaftliche Tagung der Gesellschaft für Versuchstierkunde GV-SOLAS 2011, Dresden

Vogel J, Schuler B, Arras M, Gianella M, Vogel O, Jirkof P, Cesarovic N, Klohs J, Jakob P, Baum O, Rieger G, Gubser M, Hoppeler H, Samillan-Soto V, Gassmann M, Fischer J, Born W (2012) Endogenous alpha calcitonin gene-related peptide (aCGRP) promotes physiological, exercise-induced cardiac hypertrophy. 91. Jahrestagung der Deutschen Physiologischen Gesellschaft, Dresden, 2012 (**Poster award**)

Fleischmann T, Jirkof P, Cesarovic N, Rettich A, Arras M (2012) Assessment of postoperative pain by nest complexity scoring in mice. 12th Day of Clinical Research, Zurich, 2012

Jirkof P, Cesarovic N, Rettich A, Arras M (2012) Recovery supporting effects of postsurgical group housing in female mice. 14th World Congress on pain, Milan, 2012

Nicholls F, Fleischmann T, Jirkof P, Cesarovic N, Arras M (2012) Assessment of postoperative pain by nest complexity scoring in mice. Joint symposium BCLAS/ESLAV/ECLAM, Liege, 2012. (**Poster award**)

Jirkof P, Cesarovic N, Rettich A, Fleischmann T, Arras M (2013) Individual housing of female mice: influence on postsurgical behaviour and recovery. 13th Day of Clinical Research, Zurich, 2013

Jirkof P, Cesarovic N, Rettich N, Arras M (2013) Housing of female mice in a new environment and its influence on post-surgical behaviour and recovery. ScandLAS 2013

Jirkof P, Fleischmann T, Cesarovic N, Rettich A, Vogel J, Arras M (2013) Assessment of post-surgical distress and pain in laboratory mice by nest complexity scoring. ScandLAS 2013

Jirkof P, Cesarovic N, Rettich A, Fleischmann T, Arras M (2013) Influence of housing conditions on behaviour and recovery of female mice following surgery. 12th FELASA-SECAL Congress, Barcelona, 2013

Invited Talks

"Refinement of anesthesia & analgesia in laboratory mice", University of Zurich, Institute of Evolutionary Biology and Environmental Studies, 2010.

"Surgical impact on social behaviours in mice" FELASA 2010, Helsinki, LAS Competition **2nd Runner up prize**.

"Streitpunkt CO_2 Euthanasie von kleinen Labornagern: Wo stehen wir heute?" 13. Fortbildungsveranstaltung der IGTp 2011, Dresden.

"Umweltverbesserungen für Labornager. Bericht aus der Arbeitsgruppe Labortierhaltung der GV- SOLAS / Beispiele aus der Praxis". Fortbildungskurs für Tierpflegerinnen und Tierpfleger "Beschäftigung—Tiere in Aktion" Oktober 2011, Olten.

"Monitoring burrowing and nest building behavior as species-specific indicators of animal wellbeing", Measuring Behaviour 2012, Utrecht 2012.

"Euthanasie von Mäusen; neueste Erkenntnisse und ihre Relevanz für die tägliche Arbeit", 5. Fortbildungskurs für Versuchstierpfleger/innen, Zürich 2012.

“Nest building and burrowing”, SGV Meeting 2012, Zürich, 2012.

“Euthanasia of laboratory animals” DAAD Conference “The Establishment of the first Institutional Animal Care and Use Committee (IACUC) in Egypt“, International Academy RWTH Aachen, 2013.

Grants
DAAD grant for diploma thesis (2005)

PhD scholarship of UBS foundation (2009-2012)

Travel grant of World Congress on Alternatives and Animal Use in the Life Sciences, Montreal (2011)

PhD scholarship of BVET, Switzerland (since 2012)

Awards
Prix Jean-Pierre Mieville for Animal Welfare Research, 2013